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MÜLLER'S SERODIAGNOSTIC METHODS

ROSS C. WHITMAN B.A., M.D.

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Müller, Paul Theodor

MÜLLER'S SERODIAGNOSTIC METHODS

AUTHORIZED TRANSLATION FROM
THE THIRD GERMAN EDITION

BY

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WITH 7 ILLUSTRATIONS IN TEXT

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TRANSLATOR'S PREFACE

Professor Müller's remarkably clear presentation of the complicated methods embraced under the term "Sero-diagnosis," cannot fail to find a welcome among the rapidly increasing number who are entering this field of work in America. The Translator has added the following methods, which have appeared since the German edition was published—the Hermann and Perutz method for the diagnosis of syphilis; the meiostagmin reaction of Ascoli and Izar; the cancer diagnosis of Freund and Kaminer; the cobra venom reaction in syphilis of Weil; the cobra venom reaction in cancer of Kraus, Graff, and Ranzi; the complement deviation tests of von Dungern, Schwartz and McNeil, and of Hammer, for cancer, gonorrhœa and tuberculosis respectively. Certain other additions of a minor character, and certain suggestions offered by the experience of the Translator, have been included in brackets. With these exceptions the translation follows closely the German original. The Noguchi modification of the Wassermann Reaction has been omitted at the request of its author. All apparatus and equipment mentioned in the text, which is available on the market, can be obtained from the Kny-Scherer

Company, New York, and from other dealers in laboratory supplies. I desire, also, to express my sincere appreciation of the kindness of the publishers in permitting me to make changes in, and substantial additions to, the text, down to the very moment of going to press.

Ross C. Whitman.

Denver, February 15, 1913.

PREFACE TO THE SECOND EDITION

This collection of the more important methods of serum diagnosis, originally made for the use of my students, may perhaps be welcome to many who dislike the labor of collecting the data scattered through the literature. In accordance with the purely practical purpose which suggested the work, theoretical considerations have been entirely omitted. The chief emphasis has, on the contrary, been laid on *making the description of the various methods as exact as possible, and especially on giving a complete and detailed list of the reagents and apparatus required for each test.* With a definite purpose in view, certain methods have been included, the permanent worth of which has not yet been demonstrated; for it is precisely these methods which deserve an especially thorough testing out, under the most varied conditions. Additions to the second edition are: a chapter on the preservation of sera; the determination of meat adulteration according to the method of Uhlenhuth, Weidanz, and Wedemann; and the floccule forming reactions, especially floccule forming tests for syphilis with lecithin, and with sodium glycocholate, and Klausner's reaction; Moro's alexin test; and finally, of the various

modifications of the Wassermann reaction, I have given those of Täge, Hähne, Bauer, and Tschernobugoff.

Absolute mastery of sero-diagnostic methods can, of course, only be acquired by practice and experience. I shall rejoice if this booklet serves to lighten the labor of the beginner in this field.

Paul Th. Müller.

PREFACE TO THE THIRD EDITION

The third edition of this booklet, coming as it does, so soon after its predecessor, can of course, differ but slightly from the latter. The following methods have been added: the improved and simplified test for syphilis of Stern, the Much-Holzmann "Psycho-reaction"; and the estimation of the antitryptic power of the blood according to the method of Müller and Jochmann (modified by Marcus), and according to the method of Bergmann and Meyer. The entirely proper wish of one of the book's critics, that the methods which have not justified themselves, might be mercilessly eliminated from future editions, cannot yet be complied with, since the experience needed to determine the value of such methods is still all too slight.

Paul Th. Müller.

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Müller's Serodiagnostic Methods

Methods of Injecting Animals.

Intraperitoneal Injection of Rabbits to produce Immune Serum (Method of Uhlenhuth).—An assistant grasps the rabbit's hind legs with his left hand, and the forelegs with his right, and holds the head down. Thus the intestines fall as far as possible into the upper portion of the abdominal cavity. The belly wall is turned toward the operator, who removes the hair from the lower part of the belly with scissors, cuts through the skin, which is raised for the purpose, with scissors sterilized in the flame and cooled, exposing a small area of the underlying muscle. The muscular coat is then carefully punctured with a *dull* trochar, the syringe adjusted, and the desired fluid injected. After the withdrawal of the trochar, the small wound is closed with cotton and collodion. If no assistant is available, the animal may be fastened to one of the usual animal holders.

Intravenous Injection of Rabbits.—Long-eared animals are best adapted for injections into the

marginal vein of the ear. Short-eared animals often occasion difficulty, especially to beginners. First remove the hair from the site of the injection with scissors, then rub the entire ear briskly with a bit of cotton wet with ether, or the ear may be covered with cotton soaked in hot water, until the ear vessels become distinctly distended with blood. It is still better to stroke the ear from the base outward with an electrode during the entire procedure. Insert the needle, attached to the syringe, superficially alongside the vein, and carry it forward (toward the heart) for a short distance, to make sure that the point is really within the vessel lumen, and then inject slowly, under very slight pressure. Care should of course be taken to see that no air is injected. It is a good plan to place a spring hæmostat over the needle during the injection. Being held by the needle, it does not, of course, compress the vessel until the needle is withdrawn, when it at once compresses the vessel, preventing the escape of the injected material, as well as of blood.

Methods of Drawing Blood.

Drawing Blood from the Marginal Vein of the Rabbit's Ear to Obtain Small Amounts of Serum.— For this purpose also, long-eared rabbits are best adapted, since it is often impossible to obtain from short-eared animals more than a very small

amount of serum. The vein is first dilated as described on p. 2, and is then incised with scissors or a sharp scalpel, as close as possible to the rabbit's head. As the blood drops from the wound, it may be caught either in a small test-tube, or, if only a fraction of a cubic centimetre is required, in a capillary tube (v. *a*, p. 7). The maximum amount obtainable by this method is about 10 c.c., or as much more, under favorable circumstances. To stop the bleeding, a bit of cotton may be laid on the wound and held in position with a spring forceps, which usually can be removed in a few minutes. A great advantage of this method lies in the fact that the same animal may be used repeatedly, in this way, to obtain serum.

To separate the serum from the blood proceed as follows: If the blood has been received in capillary tubes, these are treated as described on p. 9. If, on the contrary, test tubes were used, the latter may be either:

(*a*) Set aside for 24 hours at from 10 to 14° C. (*e.g.*, placed in running water), when the serum which separates from the clot is transferred to a second tube; or,

(*b*) After coagulation, the clot may be liberated from the sides of the tube with a platinum wire, the whole strongly centrifugated, and the serum thus separated immediately.

As blood-cells easily become mixed with the serum when the latter is poured from a tube, it is

often necessary to centrifugate the serum a second time, and then carefully decant the serum from the sediment. The serum may be removed after the first centrifugation by means of a pipette, the point of which is bent upward.

Drawing Blood from a Rabbit to Obtain Large Amounts of Serum.—(*a*) The animal is deeply chloroformed and placed in an animal holder; the breast and abdomen are then rubbed with alcohol, to lessen the contamination of the blood by hair; the soft parts are cut from both sides of the breast wall by a longitudinal incision, and the anterior breast wall is removed. Before the heart stops completely, a free incision is made into the heart chambers, and the animal is allowed to bleed to death into the pleural cavity, the lungs in the meantime having been removed for this purpose. The blood is transferred from the pleural cavity to a sterile graduate cylinder with a pipette having a broad lower opening. The blood may be left in the cylinder for 24 hours, at 10–15° C., or may be placed at once in test tubes, and, after coagulation, centrifugated. The further treatment is described above.

(*b*) The following method may also be employed: The animal being fastened down or held by an assistant, the operator grasps a large longitudinal fold of skin on the neck, and cuts it off with a single strong stroke of the scissors. The neck muscles, and below these the trachea, are

next exposed for a considerable distance. With forceps and scissors, the muscles are dissected away, and the carotid is exposed. This having been separated by dull dissection, two small spring hæmostats are placed on it close together, and the vessel is severed between them. The proximal end is then held with the forceps within the mouth of a sterile, wide-mouthed vessel. The wall of the carotid is now incised with fine scissors proximal to the forceps, and the blood is allowed to flow into the vessel. The yield of blood may be somewhat increased by pressing on the animal's abdomen and thorax. The further treatment is as given on p. 4.

(*c*) Finally, a third method of obtaining blood, that of Heim, is as follows:

To obtain blood which is certainly sterile, prepare a glass tube about 25 cm. long by 16 mm. diameter, bent in an obtuse angle and drawn out several centimetres at its lower end, which is finally drawn out to a capillary point. The upper end is plugged with cotton, and the whole sterilized. After the animal has been fastened to the holder, the carotid is exposed, clamped with a small artery clamp, and a ligature is passed around it proximal to the clamp. The capillary end of the tube above described is then broken off, and the end is inserted into the artery, pointing toward the heart. Care should of course be taken to see that the tube is

not thrust completely through the artery and out again on the opposite side. The ligature is then tied by an assistant, so as to include the tube. Under favorable conditions the blood will flow readily into the tube. Any clots which may form may be removed by careful suction over the cotton plug. About 60 c.c. may be obtained in this manner from a large animal. If a less amount is desired, the tube is removed earlier, the artery being ligated, first on the peripheral and then on the central side.

The tube filled with blood may be sealed at its lower end with sealing wax and then centrifugated in order to separate the serum, or it may be left in a cool place for 24 hours.

Methods of Obtaining Blood from Other Animals.—(*a*) The most convenient method of obtaining blood from larger animals, such as the horse and goat, is to distend the jugular vein by means of a rope drawn about the neck, and then to open the vein with a sterile trochar, first having shaved and thoroughly disinfected the overlying skin. (In the goat, the large vein on the inner surface of the hind leg may be reached even more easily.—*Translator.*)

(*b*) Sterile blood may also be obtained easily from a newly slaughtered animal. The first flow of blood is discarded, and the remainder is collected, under strict asepsis, in sterile glass cylinders of about 600 c.c. capacity and 6 cm. diameter. After

the clot separates the serum is removed with sterile pipettes of 50 c.c. capacity, and is preserved in the ice box, in rather wide test tubes. A more complete separation of the serum may be secured by placing a sterile weight on the clot during the coagulation. If there is any danger of contamination, the serum must be filtered through a sterile Berkefeldt filter.

Chicken's blood may be obtained most easily from the wing vessel, lying on the inner aspect of the wing; or it may be obtained from the carotid, care being taken to avoid the œsophagus and trachea.

Obtaining Small Amounts of Human Blood-Serum.

For obtaining small amounts of blood-serum, such as is required for the Widal reaction and most other serum reactions, the following method is recommended:

Method of Pröscher.[1]—*Apparatus.*—(*a*) U-shaped, or simple straight tubes of thin glass, of about 2 mm. internal diameter, and drawn out to a point at one end. The length of the tube or "capsule" is regulated by the size of the centrifuge to be used later.

(*b*) Alcohol-ether and cotton.

---

[1] Zentralbl. f. Bak., etc., vol. 31 (1902).

(*c*) A sharp scalpel, preferably double-edged, or a lancet with a platinum-iridium point, actuated by a spring.

(*d*) Sealing wax or some other material suitable for sealing.

(*e*) A file or a glass cutting knife.

(*f*) Chloride of iron, for use as a styptic, will be found convenient.

Technique.—First clean the lobe of the ear with alcohol-ether. An incision about 1 cm. long and fairly deep is then made on the outer border, near the tip. The blood should not be allowed to drop off, this being prevented by sponging with a dry clean cloth. Apply one of the tubes above described to the drop of blood, or lay it directly in the incision. The blood is drawn into the tube by capillarity. If one tube becomes plugged, another is quickly substituted. With patience and some practice a sufficient quantity of blood can always be obtained. If the bleeding is too free, it can always be checked easily by applying a sponge wet with chloride of iron. The desired quantity of blood can also be obtained easily from the finger-tip, by using a tourniquet, or by first swinging the arm vigorously for a moment. After filling, the tubes are sealed at each end with wax. It must be remembered, however, that if the glass is wet, the wax will not stick to it. If this is the case, the outside of the tube may be warmed *carefully* till dry. The sealed tubes can be mailed if

necessary. The serum is finally separated from the clot by placing the tubes in centrifuge tubes, the bottom of which is covered with cotton, and centrifugating. The waxed ends are then cut off with the glass cutter, and a mark is made with the file at the boundary line between the serum and the clot. The tubes are broken at this point, the serum being prevented from escaping by capillary attraction. Should the clot, as may happen, cling so tightly to the wall of the tube that the serum fails to separate, it should be loosened with a slender platinum wire, and the centrifugation repeated. The tube may then be opened at the boundary line as above described. The serum is then drawn directly from the tube into the measuring pipette, by applying the former, held horizontally, to the point of the latter, held vertically. In this manner any loss of serum is prevented, again by capillarity. Care must be taken to see that there are no bubbles of air in the mouth of the tube, which might pass over into the pipette; such bubbles are easily removed by touching the end of the tube with filter paper.

Method of Czaplewski-Schottelius.[2] — Instead of the customary thin-walled glass tubes, Czaplewski uses small, thick-walled centrifuge tubes, with a pointed lower end (length 5.5 cm., diameter above 0.8 cm., below 0.4 cm.). The

[2] Münch. med. Woch. (1906), p. 508.

tube is provided with a tightly fitting cork, through which passes a long, stout insect needle. The point of the needle is bent in a hook, and covered with the best fat-free, purified absorbent cotton. A small portion of the cotton extends beyond the point of the needle. (These tubes can be obtained from Theo. Schröter, Leipsic-Connewitz.)

Thoroughly soak the cotton with the blood, as it issues from the incision. The cork is then replaced, and the tube may be mailed, if desired. Centrifugate to obtain the serum. A surprisingly large yield may be obtained. The serum is removed from the tube with a pipette. This method is simpler and calls for less skill than Pröscher's method. The only equipment needed, beside the tubes above described, is a scalpel, tincture of iodine, cotton, and chloride of iron.

Obtaining Larger Quantities of Human Blood.

Larger quantities, according to Uhlenhuth, can be most easily obtained in one of the following ways:

Placental Blood.—As soon as the child's head presents, sterile towels are spread under the mother from the buttocks to the knees. The attendant, wearing sterilized rubber gloves, places the infant on these towels. The cord is then severed, and the placental end is compressed. Carefully insert the end of the cord in the mouth of a

large, sterile, glass flask or cylinder, after flaming the latter's lip. Then, by pressure on the uterus, squeeze out as much of the blood present in the placenta as possible. The flask is then closed with a sterile cotton plug. 20 to 30 c.c. may be obtained from each placenta in this way.

The use of the **Heurteloup** cupping apparatus, such as is employed by ophthalmologists, is also recommended. This yields about 15 c.c. of fluid blood.

Veni-Puncture or Phlebotomy.—The upper arm of the patient is compressed with a rubber bandage, so as to distend the veins. Rub the skin vigorously with a bit of cotton wet in alcohol, and pass a hypodermic needle into the vein at the bend of the elbow. As the blood drops from the needle it is received in a broad test tube. (The rubber bandage should always be removed *before* the needle is withdrawn from the vein.—*Translator.*) After the needle is withdrawn the opening is closed with adhesive plaster.

Finally, the **blood of cadavers** can be used, according to Ziemke, provided it can be obtained sterile.

Method of Obtaining Defibrinated Blood and Red Blood-Cells.—For this purpose, those methods of drawing the blood, in which it flows rapidly and in a full stream are preferable. The blood is received in thick-walled glass vessels, containing glass or porcelain beads (these are apt to break

and become pulverized.—*Translator*), or steel shavings, or steel ball bearings or bits of glass rod. The vessels used may be bulbs, cylinders, salt bottles, etc. One should be careful to see that they are not filled too full. They should be closed with wood or rubber stoppers, not with cotton. After the blood is placed in the bottle, the latter is shaken vigorously for at least five minutes.

To free the blood-cells of serum, the defibrinated blood is diluted with nine parts of 0.86 per cent. sodium chloride solution, and centrifugated. The clear supernatant fluid is then poured off, an equal amount of fresh salt solution is added (mixed thoroughly.—*Translator*), and the centrifugation is repeated. This process, called "washing the red blood-cells," is repeated at least three times.

The red blood-cells may also be cleared of serum without defibrinating, by receiving the blood in some fluid which prevents coagulation. For this purpose one may use either, (*a*) a 1.5 per cent. solution of sodium citrate, or (*b*) an 0.86 per cent. solution of sodium chloride, to which 0.1 per cent. to 0.5 per cent. of potassium oxalate has been added.

Inasmuch as many sera (especially those derived from the sick or from immunized animals) coagulate with extraordinary rapidity, it is advisable to bring the blood as quickly as possible in

contact with the greatest possible amount of these coagulation-preventing fluids. Thus, for example, nine parts of the fluid may be placed in a graduated cylinder, the blood allowed to flow in to the mark 10, and the contents thoroughly mixed at once. By centrifugating and washing three times with the usual 0.86 per cent. sodium chloride solution, the serum or plasma is removed at the same time with the coagulation-preventing substance.

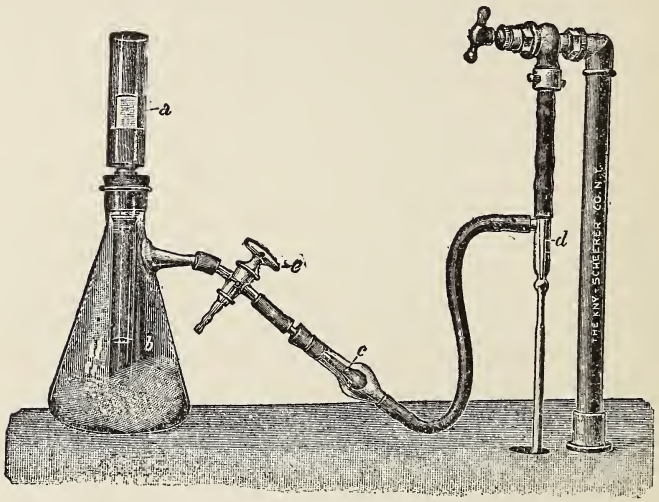
The Preservation of Sera.

Sera of Normal Animals.—Normal sera, destined to be used for purposes of immunization, are best kept in well-corked medicine bottles holding about 100–200 c.c. One may add 0.5 per cent. carbolic acid (*i.e.*, 1–10 volume of 5 per cent. carbolic acid solution) or 1 per cent. chloroform or toluol as a preservative. If either of the last two is used the serum should be shaken vigorously from time to time to aid the solution of the antiseptic in the serum. At the point of contact between the serum and the chloroform or toluol a whitish cloud forms, consisting of precipitated albumin. This usually does not extend further. The bottle should be kept in a cool, dark place.

Sera to which chloroform or toluol has been added may be placed in an open shallow dish in the incubator, for a short time before being injected, in order to remove the preservative.

Recently a preservative has been recommended consisting of a mixture of 2 c.c. of formalin with 100 c.c. of 0.9 per cent. NaCl solution. This is mixed with the serum in a proportion of 1:4, 1:3, or 2:3.[3]

Fig. 1.



IMMUNE SERA.

Immune sera may be preserved either in the fluid or in the dry form.

Preservation in Fluid Form by Means of Antiseptics.—Agglutinating sera retain their activity

[3] Leers, Methoden und Tecknik der Gewinnung, Prüfung, und Konservierung des zur Eiwessdifferenzierung dienenden Antiserums, Berlin, 1908, R. Schoetz.

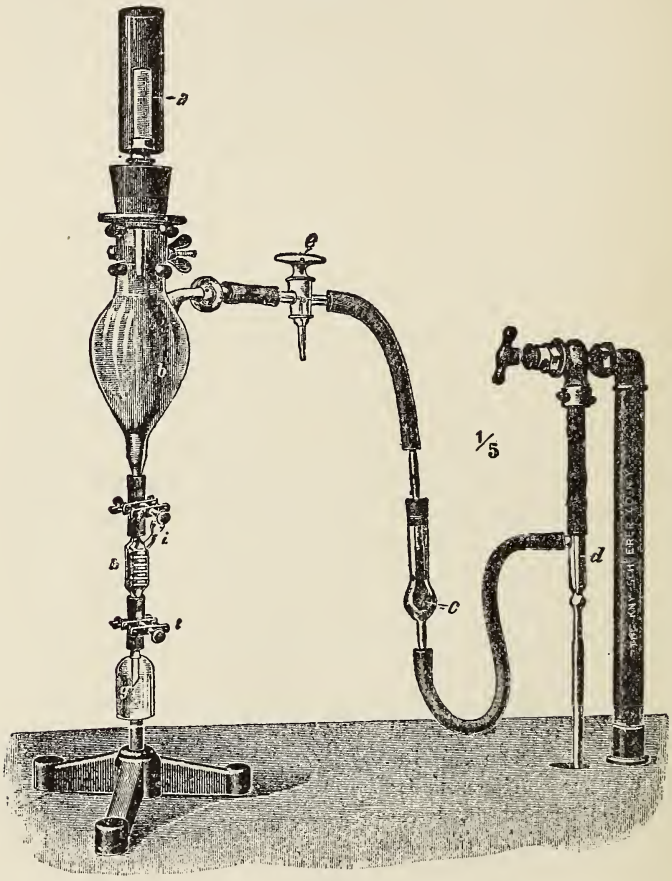
practically unchanged in the presence of from 0.3 per cent. to 0.5 per cent. carbolic acid solution. Therefore they are usually preserved in this manner. Precipitating sera may be preserved in the same way, or by the addition of a small amount of toluol (1 per cent.). According to Uhlenhuth, Weidanz and Wedemann [4] it is still better to filter such sera through a sterile Berkefeldt filter (see Fig. 1) and to store them without any antiseptic.

Preservation in Fluid Form by Germ-Free Filtration.—In a negative pressure flask (Fig. 2, *b*) connected by a rubber cork with a Berkfeldt filter (*a*) is placed a sterile test tube, into which the serum drops after filtration. The long side arm of the filtration flask is connected with a water pump (*d*) by a tube in which is a glass valve (*e*) and a back pressure valve (*c*). When the serum has all passed through, the rubber stopper is removed, the test tube lifted out with sterile forceps, and the end closed with a scorched cotton plug. The test tube is allowed to stand for some days, at room temperature, to make certain whether the filtration needs to be repeated. If the serum proves to be sterile, it is transferred with a sterile pipette into small brown vials of 1 c.c. capacity. These vials are closed temporarily with cotton only and allowed to remain for some days at room temperature. They are finally carefully sealed

[4] Arb. aus dem Kaiserl. Ges.-Amt., vol. 28 (1908).

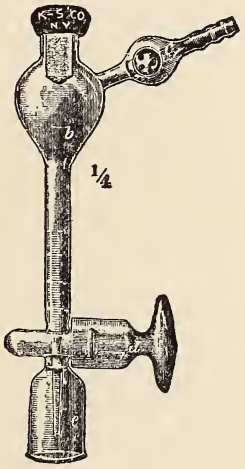
hermetically, but not until one is convinced that they are sterile by the fact that the contents remain clear.

FIG. 2.



Uhlenhuth and his collaborators have designed a special apparatus for handling the fluid, in order to prevent with certainty contamination of the serum, which may easily occur after this sort of filtration. The apparatus is a combination of a bacterial filter and a serum filling-pipette, so

Fig. 3.



arranged as to carry the filtered serum directly into the small brown vial. The apparatus may be obtained from the Kny-Scherer Co., New York (Fig. 2). Uhlenhuth and Weidanz have designed a special apparatus of small size, "Mikrofiltrier-abfülapparat" (Fig. 3), for the filtration of very small quantities. Detailed directions for the use of both pieces of apparatus are given in the article

by Uhlenhuth, Weidanz, and Wedemann, already cited.

The immune serum thus obtained is best kept in the ice box. It should at least be protected from light and heat. In case the serum is stirred just before use, the fine precipitate which often develops after long standing, may be removed by centrifugating or by filtering again. Immune sera thus preserved retain their activity for months and years.

Preservation in Frozen Condition.—In any case in which the usefulness of the serum does not depend upon its remaining absolutely clear, it may also be preserved in a frozen condition. An ice machine capable of maintaining 0° is found only in large and well-equipped laboratories. For smaller laboratories the smaller freezing apparatus devised by Morgenroth and manufactured by F. & M. Lautenschläger, in Berlin, may be adapted to meet this requirement. It is called "Frigo." A home-made freezing apparatus may be made as follows: A large kettle, holding several litres, is placed in a large wooden chest; the space surrounding it tightly packed with sawdust, and a sheet-metal covered box is placed within the kettle, to hold a small serum flask. The kettle is then filled with ice or snow, around the metal box, so that a freezing mixture is formed which will last for some days, if the chest is covered over

with several layers of felt; but it must be renewed every few days.

Freezing the serum often renders it cloudy or causes precipitates.

Preservation in Dry Form.—The serum may be spread out in the thinnest possible layer on large glass plates and dried in the incubator. After a few hours the dried serum, which adheres only slightly to the glass, can be removed with a spatula and stored in hermetically sealed tubes *in vacuo*.

It is better to carry out the drying process also *in vacuo*. The large serum institutes employ a specially made serum drying apparatus; in the smaller institutes, however, the ordinary vacuum apparatus may be adapted to this purpose. One may prepare the simplest form of drying apparatus according to the following method of Taege:[5] A glass bell jar, with a ground base and a large opening at the top, is placed on a polished iron plate which can be heated with a gas burner or set in a water-bath. The opening at the top is closed with a three-hole rubber stopper; one hole carries a thermometer, a second is connected with a manometer, while the third carries a bent glass tube connected, by means of a thick walled rubber tube, with a water vacuum pump.

The dried serum should be dissolved in physiological salt solution for use.

[5] Münch. med. Woch. (1908), No. 33.

It is simpler and more convenient to dry on paper the amount of antiserum required for a single test, according to the method of Leers,[6] since it is difficult to measure exactly the minute amounts of serum often needed for the application of biological reactions. The paper must be fairly firm in order to prevent drops soaking through. Black *Naturpapier* (hat-box paper,—*Translator*) is convenient for this purpose. With a graduated pipette 0.1 c.c. of the serum is spread in a large drop on small pieces of paper; after remaining 2 to 4 hours in the incubator, the drop is dried to a firm, shining crust. This clings firmly to the paper, on which individual portions of the serum are preserved in an exsiccator protected from light, heat, and moisture. Antiserum thus preserved retains its activity for a long time unaltered.

When a test is to be made the bit of paper carrying the single portion of serum required is washed for a short time in sodium chloride solution to remove dust and lint, and is then placed in a measured amount of salt solution; or it may be placed directly in the proteid solution to be tested, in such a manner that the paper side lies next to the solution. When the paper becomes soaked it sinks to the bottom, or it may be pushed

[6] Methoden und Tecknik der Gewinnung, Prüfung, und Konservierung des zur forensischen Blutdifferenzierung dienenden Antiserums, Berlin, 1908, R. Schoetz.

down. One should, however, avoid forcing the paper under the surface of the fluid before it is soaked, as in this case the air is not completely removed from the meshes of the paper, and the solution of the serum is affected.

The paper selected to dry the serum on should be one which does not give off much color to the solution within the time required to dissolve the serum.

(Stökel[7] recommends mixing the serum with anhydrous sodium sulphate, 1 c.c. serum to 0.7–2.0 grammes of the salt. The water of the serum is absorbed, leaving a solid which can be pulverized. By dissolving an aliquot portion (1.7–3 grammes) in 100 c.c. distilled water, an isotonic solution is obtained which represents a 1 per cent. solution of the serum.—*Translator.*)

Preservation within the Living Animal.—It is sometimes an advantage to remove only so much serum from the immunized animal as is needed for the test, without killing it. The animal may then be re-immunized from time to time, or it may be permitted to rest. Each time the animal is bled, the titre of the serum may be raised as desired by giving one or two injections. A disadvantage of this method lies in the marked tendency shown by immunized animals to sicken, or

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[7] Wien. klin. Woch., 23 (1910), No. 43.

to die with symptoms of anaphylaxis or marasmus. A larger number of animals must therefore be kept immunized, thereby adding materially to the expense.

Technique of the Serodiagnostic Methods.

precipitin reactions.

Principle.—The mixing, in suitable proportions, of an immune serum of high potency with its homologous proteid antigen causes the formation of a specific precipitate.

Practical Applications.—The identification of proteid substances of unknown origin, as, the differentiation of human and animal blood (according to Uhlenhuth), or the recognition of meat substitutions, etc.; the sero-diagnosis of syphilis.

Differentiation of Human and Animal Blood or Flesh.—*Apparatus.*—(*a*) A highly potent and absolutely crystal clear *serum immune* against the proteid to be recognized. It is best obtained by means of intravenous or intraperitoneal injection of a considerable number of rabbits with blood, or, still better, with serum. The injections should be repeated four or five times at intervals of five or six days.

Not all the rabbits injected with a foreign blood or serum will furnish a sufficiently potent immune serum. From the third injection on,

frequent tests should be made, with 2 or 3 c.c. of blood taken from the ear vein, to determine whether the rabbits' sera have attained a sufficiently high titre. If the serum tested shows the desired characteristics, the animal is killed and its serum collected. The serum must possess the following properties:

It must be absolutely clear. This can be secured by filtering through a sterile Berkefeldt filter. Further, it must not show a milky opalescence by transmitted light. Such an opalescence depends upon the stage of digestion in the animal at the time it is killed. Since it can not be removed by filtration, as is possible with precipitates, an opalescent serum must in every case be discarded. Animals whose blood when removed for the purpose of making a test shows opalescence should not be killed until the opalescence has disappeared. This usually occurs after several hours' fasting.

Finally, the serum must be of high potency and must react promptly. In order to make certain of this, an accurate titration of the serum is made before the animal is killed, and again after the final preparation of the serum. This titration is made in the same manner as when carrying out the test, as described later. For the present, therefore, only the quantities of reacting substances to be used for this special purpose are given:

Dilutions of 1–1000, 1–10,000, and 1–20,000, respectively, of the serum or blood against which the immune serum acts are first prepared with physiological salt solution (0.8 to 0.9 per cent.). To 2 c.c. of each of these dilutions add 0.1 c.c. of the immune serum, without shaking. In the 1–1000 dilution a distinct clouding should appear at once, or, at the most, after one or two minutes; and, after three to five minutes, the beginning of the reaction should be distinctly recognizable in the higher dilutions. The reaction should be fully complete after thirty minutes at the most, at room temperature.

The immune serum may be preserved in sterile, hermetically sealed glass tubes, after filtering through a sterile Berkefeldt filter. The serum will be rendered free of bacteria in this way, provided the filter works properly. Care should be taken to avoid using cracked filters.

A safer way is to preserve the serum, unfiltered, by the use of 0.5 per cent. carbolic acid (1–10 volume of 5 per cent. carbolic acid solution) as a preservative.

(*b*) A solution of the *unknown substance.* Physiological salt solution only should be used as a solvent and diluent for blood-stains, etc. The dilution should be about 1–1000. In the case of blood-spots this is not always easy to estimate. Uhlenhuth gives the following directions for obtaining the proper dilution:

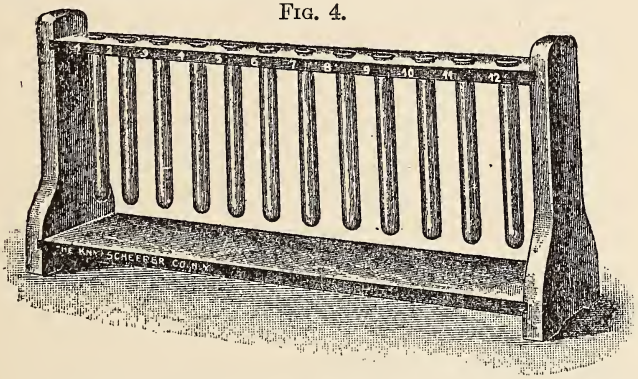
1. The dilution should be almost completely colorless by transmitted light.

2. It should give only a slight cloud on heating with a few drops of nitric acid.

3. It must foam freely on shaking, in spite of the high dilution.

This solution also must be crystal clear, for which purpose filtration may be employed.

Fig. 4.



(*c*) *Test-tubes,* 10 cm. long by 0.9–1 cm. wide. These must be absolutely clean.

(*d*) *A test-tube rack.* The rack for 12 tubes devised by Uhlenhuth is very convenient. This has the holes for the tubes bevelled, and permits the test-tubes to hang suspended in the holes. Since the small test-tubes are often of unequal diameter, it is a good plan to select in advance tubes as nearly as possible of the same size. To avoid mixing the tubes, the holes in the rack may be numbered. (See Fig. 4.)

(*e*) *Physiological salt solution.*

(*f*) *Volumetric pipettes,* 0.1 c.c. graduated in 1–100's (the calibration extending to the point), and 1 c.c. and 10 c.c. in 1–10's.

(*g*) *Dried blood-clots* from various species of animal to serve as controls. Dilutions approximating 1–1000 should be prepared of each of these in the same manner as the unknown substance.

Technique.—The following mixtures are set up in a series of test-tubes:

1. The test proper: 2 c.c. unknown solution + 0.1 c.c. immune serum.

2. Control: 2 c.c. salt solution + 0.1 c.c. immune serum.

3. Control: 2 c.c. diluted blood of that species of animal whose blood is suspected to be present in the unknown solution + 0.1 c.c. immune serum.

4. Control: 2 c.c. unknown solution alone.

5. Control: 2 c.c. diluted blood-serum of a different species of animal from that suspected to be present in the unknown solution+0.1 c.c. immune serum.

When the test proper and control No. 3 result positively, and all the others negatively, the presence of the blood or proteid of the species suspected in the unknown is established. If the result is negative, the species to which the unknown specimen belongs has to be determined with the aid of new antisera prepared for each

species, and employed in the manner above described.

No test is to be regarded as positive unless, as in the preliminary tests of the immune serum, the beginning of the reaction manifests itself within, at the most, one or two minutes after the addition of the immune serum, as a faint, misty cloud at the bottom of the test-tube; and within five minutes, at room temperature, this must become a thick, woolly cloud. At the end of another ten minutes a definite precipitate must have formed.

Any cloudiness which develops later than twenty minutes after the beginning of the reaction has no significance. It is to be noted, further, that the tubes *must not be shaken* during the performance of the test.

When only very limited amounts of the unknown substance are available, the test, according to Hauser, can be carried out in slender, scrupulously clean capillary tubes. A small amount of the unknown solution is drawn into the tube, and is then underlaid with a tiny amount of the immune serum—only so much, indeed, as will rise into the tube by capillarity. At the line of contact between the two fluids there will form a distinct grayish-white ring.

If the reaction is carried out as above described, no heterologous reactions occur. The clouding, in other words, is very strictly specific, as Nutall has shown on the basis of 16,000 blood-

tests. Only as between very closely related species—horse and ass, sheep and goats, dog and fox—can any doubt possibly arise. Beef and sheep's blood are readily distinguished.

The reaction can be obtained from blood in an advanced state of putrefaction, or from a clot which has been dried for a year.

Inasmuch as proteids give the reaction, not only blood but other proteid-containing substances can be used for the test; for example, spermatic fluid, pus, purulent sputum, urine, etc. It is well to remember this in medico-legal practice.

The application of the precipitin reaction to the detection of meat adulteration rests upon the same principle.

Recognition of Meat Adulteration (Method of Uhlenhuth, Wiedanz, and Wedermann).[8]—*Apparatus.*—(*a*) A *serum immune* against that species of flesh which is to be determined in the unknown specimen. Usually it is merely a question of the recognition of horseflesh. The serum is obtained from rabbits by immunizing them against defibrinated horse blood, or, better, horse serum. The animals are injected, preferably intravenously, every 5 days, with amounts varying from 2 to 3 c.c. Five or six days after the third injection, blood is drawn from the ear vein, and the serum which separates from the clot is

[8] Arbeiten a. d. Kaiserl. Ges. Amt, vol. 28 (1908).

titrated. The titration is carried out as follows: 1 c.c. of a 1–1000 solution of horse serum, in 0.85 per cent. sodium chloride solution, is mixed with 0.1 c.c. of the perfectly clear and non-opalescent immune serum, without shaking. If a cloudiness develops at the bottom of the test-tube at once, or at the latest after one or two minutes, the antiserum meets the requirements, and the animal may be killed and its serum separated.

Out of a considerable number of animals thus immunized, only a small part will yield a usable serum after the third injection. Rabbits whose serum yields a distinct precipitate, but whose serum is too weak, are given further injections, preferably intraperitoneally or subcutaneously. Those which show no precipitin after the third injection may be given further injections intravenously. Occasionally such animals furnish an active serum after the injection of from 60 to 120 c.c. of serum, all told.

The immune serum must fulfil the following requirements:

1. It must be absolutely clear and sterile. This may be attained by filtering through a Berkefeldt filter.

2. It must not be opalescent. To avoid this, the animal should be kept fasting for some hours before being killed.

3. It must be highly potent. The titrating of the serum, to determine the degree of its potency,

is carried out exactly as described on page 24. Its titre must reach 1–20,000; that is, it must show beginning cloudiness within five minutes, when mixed with horse serum diluted 1–20,000. Such antihorse serum may be obtained, until further notice, from the *Kaiserlichen Gesundheitsamt,* in Berlin.

(*b*) An *extract of the unknown meat.* To prepare this, about 30 grammes of flesh is removed from the deeper parts of the specimen through a fresh opening. It should contain as little fat as possible, and should be removed with a knife which has been sterilized either by boiling or in the flame. It is then placed on clean writing paper, which in turn is placed on an absolutely clean, sterile tile, or other suitable base, and is ground up, preferably with a sterile chopping knife. After being ground it is placed in a sterile Erlenmeyer flask of about 100 c.c. capacity, in which it is broken up with a sterile glass rod, and is then covered with 50 c.c. of sterile 0.85 per cent. sodium chloride solution (*not* tap or distilled water!). Salted meat may be freshened first in a large sterile Erlenmeyer flask, in which it is covered with sterile, *distilled* water, renewed several times in the course of ten minutes, without shaking.

The mixture of meat and salt solution should stand for 3 hours at room temperature, or over night in the ice box. Especially when the meat

contains a good deal of fat, a few drops of chloroform should be added to facilitate solution. To determine whether a sufficient quantity of proteid substances has passed into solution, place about 2 c.c. in a test-tube and shake vigorously. If a fine, bubbly foam develops, and persists for some time, the solution is ready for further treatment.

The extract must then be filtered perfectly clear. When the meat used is lean and fresh this is readily accomplished by a single filtration through a hard filter paper (Schleicher and Schüll, No. 575, 603, or 605), moistened with sodium chloride solution. When fat or salt meat is being examined, filtration through sterilized rotten stone is preferable.

The latter is carried out as follows: The sterilized rotten stone is worked up to a thin paste with sterile 0.85 per cent. sodium chloride solution. This is then spread out in a layer about 2 mm. thick on the filtering plate of a Buchner funnel, which has been previously carefully covered with filter paper, in order to prevent the rotten stone from passing through the funnel. For the rest, the apparatus is arranged exactly as described on page 15. A clear filtrate can be very quickly obtained with the aid of a vacuum pump. In place of the rotten stone paste, one can, of course, use the much more expensive Berkefeldt rotten stone (Kieselguhr) bougie, which must, of course, be sterilized each time before use.

A clear filtrate having been obtained, this must then be further diluted, if it contains more than 1 part of proteid in 300 parts of salt solution. To determine this, 1 c.c. of the filtrate is placed in a small test-tube and heated, and 1 drop of nitric acid, sp. g. 1.153, is added. If a marked cloud develops, which settles immediately to the bottom as a flocculent precipitate, it indicates that the solution is still too concentrated, and that it must be further diluted. At the point at which the addition of the nitric acid causes only a diffuse, opalescent cloudiness, which sinks to the bottom after five minutes as a barely perceptible precipitate, the desired concentration of the solution has been reached. Before proceeding with the test the reaction of the solution to litmus paper should be tested. It must be neutral, or only very slightly acid or alkaline. To avoid the possible addition of an excess of alkali, in case neutralization is necessary, it is convenient to use magnesium oxide rather than a soluble alkali.

(c) As controls, *extracts* of *pork* and *beef,* prepared as above.

(*d*) Perfectly clear *normal rabbit* serum.

(*e*) *Physiological salt solution.*

(*f*) *Volumetric pipettes,* 1 c.c. graduated in 1/10's, sterile.

(*g*) *Capillary pipettes.* These can be made by drawing out glass tubing 5 mm. in diameter over

a blast lamp (Fig. 5). One calibrates these by measuring into a watch crystal 0.1 c.c. NaCl solution from a slender, accurately calibrated pipette and then sucking this up into the capillary pipette. Allow the solution to drop slowly from the pipette, counting the drops. The number of drops approximately equals 0.1 c.c.

Fig. 5.

(*h*) *Test-tubes* about 10 cm. long by 0.9 to 1 cm. wide.

(*i*) *Test-tube stand.*

If the antiserum has been preserved in small, hermetically sealed tubes, and if one is not certain that the contents of the other tubes came from the same rabbit, the contents of a *single* tube

only may be used for a test, since precipitates sometimes occur from mixing antisera derived from different animals. This might lead to mistakes in the interpretation of the result.

If, on long standing, a precipitate has developed in an antiserum, this should not be stirred up, but the clear, supernatant serum should be carefully drawn off with a capillary pipette.

In handling the different solutions fresh, sterile pipettes should always be used. The rabbit serum should be added by means of a capillary pipette carefully, and in such a manner that it flows down the side of the tube and collects at the bottom. The tubes should be allowed to stand at room temperature and must *not* be shaken.

Technique.—The following mixtures are set up:

Tube 1, 1 c.c. unknown extract + 0.1 c.c. antihorse serum.
Tube 2, 1 c.c. unknown extract + 0.1 c.c. normal rabbit serum.
Tube 3, 1 c.c. horse flesh extract + 0.1 c.c. antihorse serum.
Tube 4, 1 c.c. pork extract + 0.1 c.c. antihorse serum.
Tube 5, 1 c.c. beef extract + 0.1 c.c. antihorse serum.
Tube 6, 1 c.c. sterile salt solution + 0.1 c.c. antihorse serum.

If a misty cloudiness appears in tubes 1 and 3 within five minutes, becoming denser within another five minutes, and settling within thirty minutes to the bottom as a precipitate, the other tubes remaining clear, horseflesh, or the flesh of *some other single-toed* animal is present. (Cf., p. 28.) Cloudiness which develops later than the above

should not be regarded as indicating a positive reaction. The earliest appearance of the cloud can be recognized more easily if the tubes are looked at by reflected or artificial light, with black paper or some similar substance placed behind the test-tube.

Lues Diagnosis (Method of Fornet and Schereschewsky).[9]—*Principle.*—The sera of tabetics and general paralytics give a positive precipitin reaction with the serum of syphilitics.

Apparatus.—(*a*) The perfectly clear *serum of a tabetic or general paralytic.* It is best to obtain the blood in the early morning, before breakfast. It is a good plan to filter it through Schleicher and Schüll paper No. 602.

(*b*) *Serum* of the *patient* to be tested, similarly obtained and treated. The sera may be preserved by the addition of formalin, 1–10,000, or by sealing in thin, sterile glass tubes.

(*c*) As controls, the serum of a *known syphilitic* and of a *known non-syphilitic* individual.

(*d*) *Test-tubes,* 8 cm. long by 0.5 cm. wide. It is convenient to set these in a black rack, to the back of which a piece of black cloth is attached.

(*e*) *Pipettes,* 1 c.c. in 1/100's.

Technique.—Dilutions of the sera (*a*) and(*b*),

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[9] Deutsch. med. Woch. (1907), No. 41; Münch. med. Woch. (1907), No. 30.

and of the control sera (*c*) are first made in the proportions 1–5 and 1–10. Under- or over-lay 0.15 c.c. to 3.0 c.c. of the diluted serum (*a*) with the diluted serum (*b*). The specifically heavier fluid (less highly diluted) is placed first in the test-tube with a pipette, and the lighter fluid is then allowed to flow from the pipette into the tube held on a slant.

In this manner are prepared the following combinations, the figures in parentheses referring to the degree of dilution:

a + *b*	*a* (5x) + *b*	*a* (10x) + *b*
a + *b* (5x)	*a* (5x) + *b* (5x)	*a* (10x) + *b* (5x)
a + *b* (10x)	*a* (5x) + *b* (10x)	*a* (10x) + *b* (10x)

In the same manner the controls (*c*) are under- or over-laid with the dilutions of (*a*). Tests in which the layering is not perfect must be discarded.

After 2 hours, at the most, at room temperature, a delicate ring appears in positive reactions, at the point of contact of the fluids, which becomes especially distinct if one reflects the daylight falling on the tubes by means of a piece of black paper held obliquely behind the tubes. The controls made with a non-luetic serum must remain negative, while those made with the known luetic serum must be positive, if the reaction is to have any significance.

Floccule Forming Reactions.

Principle.—In certain diseases, notably syphilis, the blood-serum develops a flocculent cloudiness when mixed with colloidal solutions of lecithin or sodium glycocholate.

Practical Applications.

Lues Diagnosis (Method of Porges and Meier).[10]—*Apparatus.*—(*a*) 1 or 2 c.c. of the *patient's blood-serum,* diluted 1–5 with physiological salt solution; and similar solutions of *known syphilitic* and known *non-syphilitic* blood-sera, to serve as controls.

(*b*) *Lecithin* (Kahlbaum), rubbed up with 100 parts of physiological salt solution to a 1 per cent. suspension, and shaken till homogeneous. 0.5 per cent. carbolic solution may be added as a preservative. The suspension remains unaltered for several weeks.

(*c*) Narrow *test-tubes,* 5 to 7 mm. in diameter.

(*d*) *Volumetric pipettes,* 1 c.c. in 1–10's.

Technique.—1 c.c. diluted serum is mixed with 0.2 c.c. of the lecithin suspension and placed in the incubator for 1 hour. The result of the reaction is a floccule formation, developing diffusely throughout the whole column of fluid, at first very fine, but becoming steadily coarser. With strongly reacting sera, an actual precipitate forms, leaving

[10] Berl. klin. Woch. (1908), No. 15.

the supernatant fluid more or less clear. The reaction may begin at once, but, as a rule, it is recognizable only after from ½ to 24 hours. The use of a lens for the observation of the reaction is, in general, to be avoided. The controls set up with normal serum must, of course, remain perfectly clear. The method appears to give practically the same results as the complement deviation test.

Lues Diagnosis (Method of Elias, Neubauer, Porges and Salomon).[11]—*Apparatus.*—(*a*) The *patient's serum* inactivated. About 0.5 c.c. is required. It must be perfectly clear. As controls, known *syphilitic* and known *non-syphilitic* sera.

(*b*) Freshly prepared 1 per cent. solution of *sodium glycocholate* (Merck).

(*c*) Small *test-tubes,* 6 or 7 mm. in diameter.

(*d*) *Volumetric pipettes,* 1 c.c. in 1–10's.

Technique.—0.2 c.c. each of serum and of the solution of sodium glycocholate are mixed and left to stand for 16 to 20 hours at room temperature. They should be disturbed as little as possible. In positive cases floccules form by the end of this period, which usually collect in the upper layers of the fluid. Cloudiness or mere traces of floccule formation is to be regarded as negative. The following modifications of the test are *not* permissible:

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[11] Berl. klin. Woch. (1908), No. 23.

1. Layering of the two reacting substances. A ring-like cloud *always* develops in this case.

2. The use of the incubator, on account of bacterial growth.

3. The use of a solution not freshly prepared.

4. The addition of carbolic acid to the solution of bile salts.

5. The use of sera which are cloudy, non-homogeneous, or which contain considerable hæmoglobin.

6. The use of special aids in judging the reaction, such as a lens or lateral illumination. The reaction is to be regarded as positive only when the floccule formation is plainly visible macroscopically. The method seems to give practically the same results as the complement deviation test.

Lues Diagnosis (Method of Klausner).[12]—*Apparatus.*—(*a*) About 1 c.c. of the *patient's blood-serum.*

(*b*) Small *test-tubes,* 5 to 7 mm. in diameter.

(*c*) *Volumetric pipettes,* 1 c.c. in 1–10's.

(*d*) *Distilled water.*

Technique.—0.2 c.c. of the serum and of the 5 and 10 fold dilutions of the same are mixed with 0.7 c.c. of distilled water, thoroughly mixed and allowed to stand at room temperature for from 2 to, at the most, 15 hours. At the end of this time, if the serum is derived from a luetic patient,

[12] Wien. klin. Woch (1908), No. 7.

there will be found at the bottom of the test-tube a precipitate of globulin, 2 to 4 mm. deep, which also often floats in part in the fluid, forming plainly visible floccules. *The reaction seems to be non-specific.*

Lues Diagnosis (Method of Hermann and Perutz).[13]—*Apparatus.*—(*a*) About 1 c.c. of the *patient's blood serum,* inactivated by heating for one-half hour at 56°.

(*b*) The following solutions:

(1) Sodium glycocholate 2.00
Cholesterin 0.40
Alcohol, 95 per cent..... 100.00

For use, this is diluted 1–20 with distilled water.

(2) Sodium glycocholate 2.00
Distilled water 100.00

This will not dissolve completely, but forms a suspension which is to be thoroughly shaken before use.

(*c*) Small *test-tubes,* 5 to 7 mm. in diameter.

(*d*) *Volumetric pipettes,* 1 c.c. in 1–10's.

(*e*) An *incubator* at 21° is convenient, but not necessary.

Technique.—Mix together one part of solution 1, diluted as described, 1 part of solution 2, and 2 parts of the serum. Shake gently and allow the tubes to stand for 24 hours at room temperature.

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[13] Medizin. Klinik, vol. 7 (1911), 60.

The formation of a *distinct precipitate* indicates a positive reaction. A slight cloudiness has no significance.

H. and P. found the test *slightly more delicate* than the Wassermann. Jensen and Feilberg [14] suggest that the tubes be allowed to stand in an incubator at 21°, instead of at the somewhat uncertain room temperature, and that the two solutions be mixed together in equal volumes just before use, and then combined in equal volumes with the serum. They found the test *slightly less delicate* than the Wassermann reaction.

The Agglutination Reactions.

Principle.—When a homogeneous, *i.e.*, uniformly cloudy, suspension of bacteria is mixed with the homologous immune serum, a precipitate develops after a short time, which consists of bacteria clinging to one another, and which sinks slowly to the bottom with clearing of the supernatant fluid. Motile species lose their motility.

Practical Applications.—The identification of unknown species of bacteria recovered from dejecta, water, etc. (the Gruber-Widal reaction); *e.g.*, the *B. Typhosus, Paratyphosus, Dysenteriæ, Bubonis, V. Choleræ Asiaticæ, D. Meningitidis*, etc.

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[14] Berl. klin. Woch., vol. 49 (1912), 1086.

The Macroscopic Reaction (Method of Pröscher).[15]—*Apparatus.*—(*a*) A *homogeneous suspension* of the bacterium to be identified; for this purpose a 12- to 24-hour-old bouillon culture is usually sufficient. Thread- and sediment-forming varieties are better grown on agar slants at 37° C., and, when 12 to 24 hours old, mixed as uniformly as possible, with a platinum loop, in a little bouillon. The coarser particles can be broken up by rubbing them against the wall of the test-tube.

The suspension should be as thick as a luxuriantly grown bouillon culture. As a measure of self-protection the bacteria may be killed by adding 0.5 per cent. carbolic acid (1–10 volume of 5 per cent. carbolic acid) before proceeding to the test.

(*b*) A highly potent *immune serum.* This is most easily obtained by giving rabbits several injections of bacteria killed by heating for one hour at 60° C. The injections may be intravenous or intraperitoneal. The interval between injections should be about 7 days. The blood is withdrawn 7 to 10 days after the last injection.

The serum may be kept in the dark, in a cool place, 0.5 per cent. carbolic acid being added as a preservative. It is better, however, to dry it *in vacuo,* and to seal it in brown glass vials containing 0.2 gramme to the vial.

[15] Zentralbl. f. Bakt., etc., vol. 31 (1902).

(*c*) Small *test-tubes,* about 10 cm. long by 9 mm. inside diameter.

(*d*) *Volumetric pipettes,* 1 c.c. in 1–100's for the preparation of dilutions; and 1 c.c. and 10 c.c. pipettes in 1–10's for the addition of the sodium chloride solution and bacterial emulsion. The calibration should extend to the point of the pipette.

(*e*) *Physiological salt solution.*

(*f*) In place of test-tubes, small *glass dishes,* such as are used for staining microscopic preparations, may be used.

(*g*) A *microscope* with dry lenses magnifying about 50 diameters.

Technique.—The following dilutions of the immune serum are prepared with physiological salt solution:

1–50 (*i.e.,* 0.1 c.c. serum + 4.9 c.c. salt solution).
1–100 (*i.e.,* 4 c.c. dilution 1–50 + 4 c.c. salt solution).
1–500 (*i.e.,* 2 c.c. dilution 1–100 + 8 c.c. salt solution).
1–1000 (*i.e.,* 1 c.c. dilution 1–100 + 9 c.c. salt solution).
1–2000 (*i.e.,* 0.5 c.c. dilution 1–100 + 9.5 c.c. salt solution).

Place 0.5 c.c. of each of these in one of the test-tubes above described, and add to each the same amount of a homogeneous bacterial emulsion. The quantity of the latter may be varied, from 0.2 to 0.5 c.c., according to the richness of the suspension. The total contents is then brought to *1 c.c.* by the addition of salt solution. This, of

course, doubles the dilution of the serum in each tube.

Or, one may transfer direct one loop of bacteria from the solid culture medium to each serum dilution, and mix as uniformly as possible.

The controls are:

(*a*) The bacterium in question + salt solution only, the total volume as above, 1 c.c.

(*b*) The highest serum dilution employed + a 24-hour culture of the bacterial species with which the unknown bacterium is sought to be identified, and which is homologous with the immune serum employed.

Place all the tubes for 1 to 3 hours in the incubator at 37° C., and examine with the naked eye, or under slight magnification. Control (*a*) should remain homogeneous, while (*b*) and the test proper should show distinct clumping if the reaction is positive.

According to Pröscher, the test-tubes containing the several mixtures of serum and bacteria may be poured into small staining dishes, and these may be nested on top of one another, set in the incubator, and, after the proper interval, examined with the microscope under a magnification of about 50 diameters, the condenser being removed for the purpose. (The fact that cultures of Typhosus, etc., recently isolated from water, stools, blood or urine are apt to be much less agglutinable than laboratory stock cultures of the

same species, renders this and the following test of doubtful value, unless great judgment is used in the interpretation of the results.—*Translator.*)

The Microscopic Reaction (Hanging Drop Method.).—When, to save time, one wishes to test colonies taken direct from a plate, the test must be made in a hanging drop, on account of the small amount of bacterial growth available. *Apparatus.*—(*a*) *Hollow ground slides.*

(*b*) *Cover glasses.*

(*c*) *Vaseline,* with which to ring the edge of the depression in the slide, and thus secure exclusion of air from the moist chamber. A brush is convenient to handle the vaseline with.

(*d*) *Immune serum* of high potency, as described on page 42.

(*e*) Physiological salt solution.

(*f*) Pipettes as described under (*d*), p. 43.

(*g*) A platinum loop and a platinum needle.

(*h*) Microscope with homogeneous immersion lens.

Technique.—One arranges dilutions of the immune serum as for the macroscopic test. For example:

1–100 (*i.e.*, 0.1 c.c. serum + 9.9 c.c. salt solution).
1–500 (*i.e.*, 2 c.c. dilution 1–100 + 8 c.c. salt solution).
1–1000 (*i.e.*, 1 c.c. dilution 1–100 + 9 c.c. salt solution).

If the amount of bacterial growth in the colony to be tested is very slight, one must be satisfied with a single dilution, viz., 1–100.

With the platinum loop, place the smallest possible drop of each of these dilutions in the centre of a thoroughly clean cover glass; with the platinum needle material is removed from the desired colony, and rubbed up to a homogeneous emulsion, till a distinct cloudiness is visible to the naked eye. (Caution: avoid scattering droplets of the emulsion.) The hollow ground slide, previously ringed with vaseline, is pressed upon the cover glass with the depression down in such a manner that the drop lies exactly in the middle of the depression. The whole preparation is then turned over. The cover glass now lies on the top, with the drop hanging in the depression. If the drop touches the lower wall of the moist chamber the drop is too large, and the preparation must be discarded.

When the serum used is very strong, agglutination, (*i.e.*, immobilization of motile varieties, and clumping) may be observed immediately after mixing the serum with the bacteria, or, at the most, after twenty minutes' incubation. Clumps found at the periphery of the drop, while the bacteria at the centre remain free, have no significance.

DETERMINATION OF THE AGGLUTINATING POWER OF A PATIENT'S SERUM (THE WIDAL REACTION).

The Macroscopic Method of Pröscher.—*Apparatus.*—(*a*) At least 0.1 c.c. of the patient's serum.

(*b*) A homogeneous suspension of the species of bacteria suspected to have caused the disease (*B. Typhosus, Paratyphosus, Dysenteriæ, V. Choleræ,* etc.). When a large number of tests is to be made, the emulsion may be prepared in large amounts by inoculating a litre, for example, of bouillon; after growing for 24 hours, it may be killed by the addition of 0.5 per cent. carbolic acid; or mass cultures may be prepared on agar in broad, shallow flasks, and after 24 hours washed off with carbolized salt solution. The emulsion is made as uniform as possible by shaking. Such an emulsion may be used for months if kept in a cold place. Since the bacteria gradually settle on standing, the emulsion should be thoroughly shaken before use. The emulsion is made more stable by adding about 10 per cent. milk sugar in addition to the carbolic acid. Emulsions which agglutinate spontaneously can not, of course, be used.

For the diagnosis of typhoid and paratyphoid fever, one may use with advantage the typhoid or paratyphoid diagnosticum of Ficker as prepared by Merck (and others, *Trans.*), which contains the essential parts of the bacterial cell, and remains usable for months. The sediment which forms on standing should be removed by shaking before use.

For the preparation of small quantities of emulsion see above (*a*), p. 42.

(*c*) Small test-tubes as above (*c*), p. 43.

(*d*) Volumetric pipettes as above (*d*), p. 43.

(*e*) Physiological salt solution.

(*f*) Small glass watch crystals.

(*g*) Microscope, with a dry lens magnifying to about 50 diameters.

Technique.—The patient's serum is first diluted 10 times. For this purpose it is drawn up in a volumetric pipette, measured, placed in a test-tube, and 9 volumes of salt solution added. A row of test-tubes is then arranged in a suitable rack. In each tube, omitting the first, place 0.5 c.c. salt solution. In tubes 1 and 2 place 0.5 c.c. of the 1–10 patient's serum. In tube 3 place 0.5 c.c. from tube 2; in tube 4, 0.5 c.c. from tube 3, etc. After each addition the tubes should, of course, be thoroughly mixed, and the volumetric pipette employed should be thoroughly rinsed out before proceeding to the next transfer, unless a fresh pipette is used each time. The tubes will finally contain each 0.5 c.c. of serum diluted 1–10, 1–20, 1–40, etc. Usually five or six dilutions are employed.

The control is a tube containing no serum, but only 0.5 c.c. salt solution.

Now add 0.3 to 0.5 c.c. of the bacterial emulsion to each tube, and, if necessary, bring all tubes to 1 c.c. by adding salt solution. The same amount of emulsion should be added to each tube. The serum dilution is thus doubled in each tube. The

following table explains how the several dilutions are reached:

	Tube No. 1.	Tube No. 2.	Tube No. 3.	Tube No. 4.	Tube No. 5.	Tube No. 6.	Tube No. 7.
Salt solution..	0	0.5	0.5	0.5	0.5	0.5	0.5
Serum, 1–10...	0.5	0.5	0	0	0	0	0
From tube No. 2	0	0	0.5	0	0	0	0
From tube No. 3	0	0	0	0.5	0	0	0
From tube No. 4	0	0	0	0	0.5	0	0
From tube No. 5	0	0	0	0	0	0.5	0
From tube No. 6	0	0	0	0	0	0.5*	0

Final dilutions after bacterial emulsion is added are 1-20, 1-40, 1-80, 1-160, 1-320, 1 640, control.

*Discarded.

The rest of the test is carried out as described on p. 43.

When the Ficker Diagnosticum is employed the tubes remain at room temperature, and should show after 10 to 14, or, at the most, 20 hours, clearing of the fluid, with the formation of a precipitate.

Experience has shown that the reaction should be regarded as positive only when agglutination occurs in dilutions of 1–40, 1–80, or higher. In doubtful cases the test should be repeated every 2 or 3 days, since the agglutinin content of the serum is usually found in increasing amounts in typhoid. In the presence of icterus no significance attaches to reactions in dilutions less than 1–100, since in this condition positive reactions have often been observed in dilutions up to 1–75, in the absence of typhoid.

Since it is especially important, for clinical purposes, to determine to which one of the typhoid-like organisms the disease is due, it is recommended that the test be carried out not only with the typhoid bacillus, but also at the same time with the types *A* and *B* of the paratyphoid bacillus, or with the corresponding diagnostica of Ficker.

Method of Kafka.[16]—In addition to the patient's serum, there are required for this method:

1. Small staining dishes to hold about 2 or 3 c.c.
2. A capillary pipette, made from glass tubing of about 5 mm. diameter, drawn out and calibrated empirically in such a manner that it divides 0.5 c.c. into 40 drops.
3. Ficker's Typhoid and Paratyphoid Diagnostica.
4. A volumetric pipette, 5 c.c. divided in 1–10's.

Technique.—Place 0.5, 1, and 2.5 c.c. respectively of each diagnosticum in the staining dishes and with the capillary pipette add 1 drop of serum to each. The serum is thus diluted 1–40, 1–80, and 1–200. Mix thoroughly by tipping the dishes back and forth, and allow them to stand at room temperature. The results are observed at the end of 24 hours. For the diagnosis of an *existing* typhoid infection (but not of an infection of some time previous), the reaction must occur in a dilution of 1–200, while the paratyphoid shows a titre more

[16] Zentralbl. f. Bakt., vol. 40 (1906).

or less lower than this. If two or more of the diagnostica are positive in the highest dilution, the serum must be further diluted, and the test repeated till a higher titre for one is found than for the other two. The following table illustrates how various dilutions may be obtained:

4 drops serum 1–10 + 0.5 c.c. diagnosticum = dilution 1–100
2 drops serum 1–10 + 0.5 c.c. diagnosticum = dilution 1–200
1 drop serum 1–10 + 0.5 c.c. diagnosticum = dilution 1–400
1 drop serum 1–10 + 1.0 c.c. diagnosticum = dilution 1–800
1 drop serum 1–10 + 1.5 c.c. diagnosticum = dilution 1–1200
1 drop serum 1–10 + 2.0 c.c. diagnosticum = dilution 1–1600
1 drop serum 1–10 + 2.5 c.c. diagnosticum = dilution 1–2000
et cætera.

The diagnosticum which reacts in the highest dilution may, with however, some reserve, be regarded as indicating the etiologic agent. If the titre for the typhoid is the same as that for the paratyphoid, or even if the latter is but slightly greater, the typhoid should still be regarded, according to Güttler,[17] as the etiologic factor. (It is doubtful if a differential diagnosis between typhoid and paratyphoid fever can be made by this or any similar method. The quantitative relation of the direct to the group agglutinins is so irregular, and the relative agglutinability of different strains of the bacteria concerned so variable, that such comparative quantitative tests can hardly amount to more than the roughest of approximations.—*Trans.*)

[17] Berl. klin. Woch. (1904), Nos. 51 and 52.

Hanging-Drop Method.—*Apparatus.*—(*a*) The serum to be tested.

(*b*) Cover glasses.

(*c*) Vaseline and brush.

(*d*) Hollow ground slides.

(*e*) Physiological salt solution.

(*f*) Volumetric pipettes, 0.1 c.c. in 1–100's, and 10 c.c. and 1 c.c. in 1–10's.

(*g*) Microscope with oil immersion lens.

(*h*) A 10- to 24-hour-old agar slant culture of the organism against which the serum is to be tested (typhoid, paratyphoid, etc.).

(*i*) Platinum needle and platinum loop.

Technique.—The technique is that given on p. 45, with the following exception:

Prepare the following dilutions of the patient's serum:

1–25 = 0.1 c.c. serum + 2.4 c.c. salt solution.
1–50 = 1 c.c. dilution 1–25 + 1 c.c. salt solution.
1–100 = 0.5 c.c. dilution 1–50 + 0.5 c.c. salt solution.
1–150 = 0.5 c.c. dilution 1–50 + 1 c.c. salt solution.

Mix a trace of the culture, by means of the platinum needle, with a drop of each of these dilutions. The controls are: (1) A drop of bouillon or salt solution + bacteria as above, and (2) a drop of known positive agglutinating serum + bacteria. When the reaction is positive, agglutination occurs in the dilutions of the patient's serum up to 1–100 or higher, and in the second control, while the first control remains unchanged.

Diagnosis of Mixed Infection by Means of the Agglutination Test (The Saturation Test of Castellani).[18]—If the agglutinating power of a patient's serum is tested against both the homologous organism and against closely related organisms (*e.g.,* typhoid and paratyphoid bacilli), an increased agglutinating power against the latter as well as the former is frequently found, although it has no share in the production of the disease. This is the so-called "group agglutination."

On the other hand, in case of mixed infection, the agglutinating power is likewise increased against the several species of organisms concerned. In order to determine, in a given case, in which a serum agglutinates several species equally, whether one is dealing with a true mixed infection, or whether the result is due to group agglutination merely, the Castellani test may be employed.

Principle.—(*A*) The serum of an individual immunized against a single species only loses its agglutinating power for *all the species* it is capable of influencing, when mixed with an excess of the homologous organism, which absorbs the entire mass of agglutinins; but when mixed with other than the homologous organism, *i.e.,* with organisms influenced only through group agglutination,

[18] Zeitschr. f. Hyg., vol. 40 (1902).

it loses its agglutinating power for these organisms only, but not for the homologous organism.

(*B*) The serum of an individual immunized against two organisms, *A* and *B*, loses its agglutinating power for *A* only, when mixed with an excess of *A;* and for *B* only when mixed with an excess of *B*.

The apparatus required is essentially that needed for the Widal test, as previously described.

Technique.—From 0.5 c.c. to 1.0 c.c. of the serum to be tested, and which, we will assume, agglutinates equally the typhoid and colon bacilli, is placed in each of two test-tubes, and mixed with relatively large amounts of the typhoid and colon bacillus respectively (4 to 8 loops of a 24-hour-old agar slant culture). The bacteria are thoroughly mixed with the serum, and the tubes then placed in the incubator for 12 hours. At the end of this time the serum, after being separated from the agglutinated masses of bacteria by centrifugation, is tested to determine whether it still contains agglutinating power for the same organism as that first added. If so, more bacteria are added, and the incubation is repeated. When the agglutinating power for the respective organism employed has disappeared (in which case the emulsion remains cloudy), the bulk of the bacteria remaining is removed by centrifugation at a high speed, or by allowing the tubes to settle for 12 to 24 hours in the ice-box. The serum is then tested for its

agglutinating power against the species which was *not* used in the first test. Thus if the typhoid bacillus was added in the first instance to tube *A*, the serum in this tube is now tested against the colon bacillus; and tube *B*, to which the colon bacillus was first added, is now tested against the typhoid bacillus.

Should the test show that absorption of agglutinins by each species leaves the agglutinating power for the other practically unchanged, the case is in all probability one of mixed infection by the typhoid and colon bacilli. If on the other hand absorption of agglutinin by the typhoid bacillus causes a material reduction in the agglutinating power for the colon bacillus, it is probably an infection by the typhoid bacillus alone, and the effect of the serum on the colon bacillus is to be regarded as due merely to group agglutination.

Practical tests of the Castellani method are not yet very numerous.

Bactericidal Reactions.

In the Living Animal (the Pfeiffer Phenomenon).—*Principle.*—When bacteria, mixed with the homologous immune serum, are injected into the peritoneal cavity of a guinea pig, solution of the bacteria occurs in a short time, with the typical granule formation; but when injected along with a normal or heterologous immune serum, they remain intact, or even actively motile.

PRACTICAL APPLICATIONS.

Identification of Bacteria Recovered from the Dejecta, Water Supplies, etc.—(The method is chiefly employed in the identification of the cholera and similar vibrios, typhoid, and paratyphoid bacilli, etc.).

Apparatus.—(*a*) Four guinea pigs, weighing from 200 to 300 grammes each.

(*b*) Immune serum of high potency. This is most easily obtained by giving rabbits one or more intraperitoneal injections of a known culture of the organism with which the unknown organism is to be identified. The organism should be grown on agar, an emulsion prepared and killed at 60° C. The animal should be killed 14 days after the last injection.

The mixed serum of several animals so treated should be dried in vacuo, and placed in small amber colored glass vials, containing 0.2 gramme each, for preservation. Such vials may be obtained from the Bacteriological Institute of the Royal Prussian Institute for Infectious Diseases.

The serum must be at least of such a strength that 0.0002 gramme suffices to convert completely within one hour 1 loop (= 2 mg.), of an 18-hour-old culture of a virulent cholera vibrio or typhoid bacillus respectively, into granules when injected, mixed with 1 c.c. of bouillon, into the abdominal

cavity of a guinea pig. In other words, its "titre" must be at least 0.0002 gramme.

(*c*) Rabbit serum, fresh, or dried and preserved in the same way.

(*d*) An 18-hour-old agar slant culture of the organism to be tested.

(*e*) A syringe with a blunt needle.

(*f*) Shears and forceps for making the incision for the injection.

(*g*) Several capillary tubes, prepared by drawing out glass tubing 0.5 cm. in diameter. These are used for removing the fluid from the abdominal cavity.

(*h*) Four funnel-shaped glasses, of the size and shape of a liqueur glass, in which to mix the fluids for injection.

(*i*) Hollow ground slides, cover glasses, and vaseline for the preparation of hanging drops.

(*k*) A platinum loop calibrated to take up 2 mg. of the bacterial growth.

(*l*) Volumetric pipettes, 0.1 c.c. in 1–100's and 1 c.c. and 10 c.c. in 1–10's.

(*m*) Sterile bouillon.

(*n*) Physiological salt solution.

Technique.—In glass 1 place five times the titre unit of the serum (*i.e.*, about 0.001 gramme of a serum having a titre of 0.0002).

In glass 2 place ten times the titre unit of the serum (0.002 gramme in our example).

In glass 3 place, as a control, fifty times the

titre unit of *normal* serum of the species furnishing the immune serum.

In order that the various amounts of serum may have like relations, they must be contained in equal volumes of fluid. The amounts of serum mentioned above are therefore prepared in suitable dilutions. In our example, 0.2 gramme dried immune serum is dissolved in 10 c.c. salt solution. Of this 0.2 c.c. representing 0.002 gramme, or 2 mg., is placed in the *second* glass. A portion of the remaining fluid is then mixed with an equal volume of salt solution, so that the 0.2 c.c. to be placed in the first glass, represents 1 mg. of serum. The required amount of normal serum is obtained by dissolving 0.2 gramme of the dried serum in 4 c.c. of salt solution.

Finally, in glass 4, place 4 c.c. of bouillon, 0.8 c.c. salt solution, and 1 loop from the agar culture. The 1.2 c.c. injected will thus contain exactly .25 loop of bacteria.

The contents of the various glasses having been thoroughly mixed, the injection is made, by incising the outer skin with the scissors, and puncturing the peritoneum with the dull needle. The first three guinea pigs receive the contents of the first three glasses respectively, plus 1.2 c.c. from the fourth glass. The fourth animal receives only 1.2 c.c. from the fourth glass.

At the end of 20 minutes, and again at the end of an hour, a drop of fluid is removed from the

abdominal cavity of each animal, through the same opening, by means of one of the capillary pipettes (usually pressure on the abdomen suffices to cause the fluid to enter the tube), and examined in a hanging drop preparation.

If guinea pigs Nos. 1 and 2 show granule formation, at the latest after an hour, while in the third and fourth animals, large masses of bacteria remain motile, and if well preserved vibrios are still present, the reaction may be regarded as positive, and the diagnosis as established.

Demonstration of Bactericidal Antibodies in the Serum of Sick or Convalescent Patients. (Diagnosis of a recent cholera infection, etc.).—*Apparatus.*—The same apparatus is required as on p. 56, except that in place of (*d*) one employs an 18-hour-old agar slant culture of the true cholera vibrio, or typhoid bacillus, as the case may be; and in place of (*b*), the serum of the patient.

Technique.—Dilutions of the patient's serum are prepared with 20, 100, and 500 parts of sterile bouillon, and 1 c.c. of each of these is placed in one of the first three liqueur glasses. To each glass is then added 1 loop of the culture.

Glass four contains 4 c.c. of bouillon without any serum, + 1 loop of culture, so that the amount injected (1 c.c.), contains .25 of a loop. The remaining technique is as above, p. 58.

If granule formation occurs in the first three animals, but is wanting in the fourth, the reaction

may be regarded as positive, and the diagnosis of cholera, etc., as established.

If granule formation occurs in the fourth animal also, the culture employed is to be regarded as unsuitable.

Bactericidal Reactions in Vitro (Determination of Bactericidal Substances in the Blood-Serum of Patients).—This method was devised by Stern and Korte,[19] for the diagnosis of typhoid fever. It is complicated, and is only rarely to be recommended, in cases in which the agglutination test has yielded uncertain results.

Principle.—The patient's serum is inactivated by heating for half an hour at 56° C., and is then mixed with a fresh complement containing normal serum, which is, *per se,* inactive. "Plating out" tests are then made to determine in how great a dilution of the serum bactericidal activity can still be demonstrated, as shown by a reduction in the number of colonies as compared with control tests.

Apparatus.—(*a*) The patient's serum, heated for half an hour at 56° C., and then diluted 50 to 100 times with sterile, 0.85 per cent. sodium chloride solution.

(*b*) Serum of a normal rabbit, obtained the same day it is to be used, and diluted 10 to 15 times, to serve as complement.

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[19] Berl. klin. Woch. (1904).

(*c*) A 24-hour-old bouillon culture of typhoid bacilli, diluted 500 times with physiological salt solution.

(*d*) Short, sterile test-tubes (about 10 cm. long), of the usual diameter, provided with cotton plugs, and arranged in a rack.

(*e*) Sterile physiological salt solution.

(*f*) Sterile Petri dishes.

(*g*) Nutrient agar, in tubes.

(*h*) Sterile pipettes, 10 c.c. and 1 c.c. in 1/10's.

(*i*) A water bath for melting the agar.

(*j*) A thermometer.

(*k*) An incubator at 37° C. This may be dispensed with if necessary.

Technique.—Place 1 c.c. of sterile salt solution in each of 12 test-tubes, arranged in a rack. Then place in the first of these tubes 1 c.c. of the inactivated and diluted patient's serum. Mix thoroughly by repeated sucking up and forcing out again of the fluid; and then (with the same pipette) draw up 1 c.c. and transfer to the second tube of the series. Mix as before, and transfer 1 c.c. from this tube to the third. Continue thus to the last tube, from which, finally, 1 c.c. is discarded.

Each tube now contains 1 c.c. fluid, representing dilutions of 1–2, 1–4, 1–8, 1–16, etc., of the *original dilution* of the patient's serum.

Now add to each tube 0.5 c.c. of the bacterial

emulsion; and finally, 0.5 c.c. of the diluted complement serum.

The controls are 1–(*a*) and (*b*) each 1.5 c.c. of salt solution + 0.5 c.c. of the bacillary emulsion; 2–1 c.c. salt solution + 0.5 c.c. diluted complement serum + 0.5 c.c. bacillary emulsion.

Control tube (1 *a*) is immediately mixed with agar, previously liquefied and cooled to 41° C., rapidly mixed, and poured into a sterile Petri dish. This plate shows the original number of colonies.

All the remaining tubes are placed for from 3 to 4 hours in the incubator, and are thereafter plated in the same manner as (1 *a*). Gelatine may be used in place of the agar if desired.

Control (1 *b*) will show how much the bacteria increased during the period of incubation, in the absence of bactericidal substances. Control 2 shows the lack of activity of the complement serum alone, in the absence of the specific bacteriolysin. If, contrary to expectation, this control shows marked bactericidal activity, the entire test is without significance. The plates, which are to be placed in the incubator *bottom up,* may be examined after as short a time as twelve hours. (Gelatine plates, which must be kept at 22°, are ready for examination only after 48 hours.)

It is unnecessary actually to count the colonies which develop, since significance can only be attached to marked and immediately recognizable

differences. As an example, an experiment of Stern and Korte, with a highly potent typhoid serum is here reprinted.

1 c.c. typhoid serum.	Typhoid culture.	Rabbit serum 1–12.	Plates poured after three hours at 37°.
1–2000.....	0.5	0.5	0–100 colonies
1–4000.....	0.5	0.5	
1–8000.....	0.5	0.5	
1–16000.....	0.5	0.5	
1–32000.....	0.5	0.5	100–1000 colonies
1–64000.....	0.5	0.5	
1–128000....	0.5	0.5	
1–256000....	0.5	0.5	
1–512000....	0.5	0.5	More than 1000 colonies
1–1024000...	0.5	0.5	Less than in control 2
1–2048000...	0.5	0.5	Less than in control 2
1–4096000...	0.5	0.5	Like control 2

Controls:

(1 *a*) 1.5 c.c. NaCl + 0.5 typhoid culture poured at once .....35,000 colonies

(1 *b*) 1.5 c.c. NaCl + 0.5 typhoid culture poured after 3 hours ..... ∞ colonies

(2) 1.0 c.c. NaCl + 0.5 typhoid culture + 0.5 rabbit serum 1–12, poured after 3 hours..... ∞ colonies

According to Hahn,[20] the sera of non-typhoid individuals show bactericidal effects in only about 1–3 of the cases. Moreover this is only very exceptionally demonstrable in dilutions above 1–500. On the other hand, the sera of patients still ill with the fever, or but recently recovered,

[20] Deutsch. Arch. f. klin. Med. (1905).

are bactericidal in dilutions greater than 1–1000, and usually as high as 1 in 50,000. (In the experiment recorded on page 48, this effect is manifest in a dilution of 1 in 2,048,000, and is marked in a dilution of 1 in 512,000.)

The higher the bactericidal titre runs above that usually found in normal sera, the more probable it is that the case is one of typhoid infection.

Cytolytic Reactions

Cancer Diagnosis of Freund and Kaminer.[21]—*Principle.*—The blood-serum of normal individuals has the power to dissolve cancer cells. The blood-serum of patients suffering from cancer lacks this power, and has the power to inhibit the destruction of such cells by normal serum.

When the blood-serum of cancer patients is mixed with a cancer extract, a precipitate forms.

Application.—The diagnosis of malignant disease.

Apparatus.—(1) An emulsion of cancer cells. Grind in a mortar the undegenerated portions of the tumor, freed so far as possible of fat and extraneous cells, with about five volumes of 1 per cent. sodium biphosphate. Squeeze the suspension through several thicknesses of gauze. Allow the cells to settle, and remove the supernatant

[21] Biochem. Zeitschr., 1910; Wien. klin. Woch., vol. 23 (1910), 378 and 1221; 24 (1911), 1759.

fluid with a pipette. Wash the residue with 0.6 per cent. sodium chloride solution. Allow the cells to settle again, pipette off the supernatant fluid and cover the residue with one per cent. sodium fluoride. The last named fluid should be first neutralized against alizarin till only a trace of violet color remains. This emulsion will keep for several weeks in the ice-box.

(2) An extract of cancer cells. Fresh tumor tissue obtained at autopsy, or tumor tissue preserved in alcohol may be used. Portions as free as possible of degenerated areas and fat are cut into small pieces and worked through a coarse meshed cloth by the gradual addition of 10 volumes of 0.6 per cent. acid sodium phosphate solution. Allow the cells to separate by settling or by careful centrifugation. Wash several times with the same fluid, and preserve at 0°. Sodium fluoride, up to 1 per cent., may be added as a preservative, provided care is taken to see that it does not render the fluid alkaline. Thymol may also be used.

For use add 5 c.c. of 5 per cent. acetic acid to 100 c.c. of the fluid. Heat the mixture in the water-bath for 15 minutes at 80°, filter, cool, and neutralize to litmus with sodium carbonate. Heat again as above, cool and filter. Heating at 100° or over the free flame must be avoided. The activity of the extract is determined by testing with 10 drops of known normal and cancerous sera

whether the extract, undiluted, and diluted 10, 50, and 100 times, gives a precipitate with cancer serum, plainly visible in test-tubes held against the window, while the normal serum gives no such precipitate. The extract keeps for only two or three days. The extract if used in too concentrated form causes a precipitate with normal serum, but if diluted to opalescence gives a distinct cloud which clears upon the addition of normal serum, or of an ether extract of horse serum.

(3) The patient's serum. This is obtained in any of the customary ways. The serum must be separated from the cells within a few hours after the blood is drawn and cannot be used when it is more than 48 hours old.

(4) A blood-counting chamber.

(5) Small test-tubes or small test dishes with parallel sides.

(6) Capillary pipettes, and volumetric pipettes, 1 c.c. in 1–100's.

(7) An incubator at 37°.

(8) An 0.5 per cent. solution of sodium fluoride.

Technique.—To 10 drops of the patient's serum add 1 drop of the 0.5 per cent. solution of sodium fluoride. Then add 1 drop of the cancer cell emulsion so diluted that when 1 drop of the mixture is placed in the blood-counting chamber, about 10 to 20 tumor cells will be found in a large field (25 of the smallest squares) of the apparatus.

Close the counting chamber carefully and place in the incubator for 24 hours. Count the number of cells again.

A material reduction in the number of cells will be found when the serum is derived from a healthy individual, whereas, if the serum is derived from a cancer patient such a reduction does not take place.

A second test is made by diluting normal serum with an equal amount of 0.6 per cent. sodium chloride solution, and also with an equal volume of the patient's serum. To each of these fluoride and the cell emulsion is added as above. If the patient's serum prevents the solution of the cells by the normal serum, this again constitutes a positive reaction.

The test for a precipitate is made as follows: Place 10 drops of the patient's serum in a small test-tube or test dish with parallel sides and add 2 c.c. of the extract properly diluted as described above. Controls are made by adding to a separate quantity of serum a fluid identical with the tumor extract with the exception of the tumor tissue. In other words, if the extract was prepared from fresh tumor tissue this fluid is prepared by adding to 100 c.c. of 1 per cent. acid sodium phosphate, 5 c.c. of 5 per cent. acetic acid, and neutralizing with sodium carbonate. When the extract is prepared from tissues preserved in

alcohol, use 0.25 per cent. acetic acid neutralized with sodium carbonate.

The precipitate forms at once and must be viewed by transmitted, not by reflected light. A distinct clouding under these conditions constitutes a positive reaction. The authors of the test recommend that all three tests should be made when enough serum is available. This calls for about 2.5 c.c. of serum, representing about 6 c.c. of whole blood.

Diagnosis of Pregnancy (Improved Method of Abderhalden).[22]—*Principle.*—The blood serum of pregnant individuals contains a specific ferment which digests placental cells.

Apparatus.—(1) *Placental tissue.* Cut a fresh human placenta into small pieces, place in a wide dish, and wash in running (tap) water. The maternal portions may be separated and discarded, but this is not necessary. While the washing, which is for the purpose of removing blood, is going on, a large enamel basin or evaporating dish containing 1 to 2 litres of water, to which 1 drop per litre of glacial acetic acid is added, is heated to boiling. Throw the washed placenta into the boiling water, and continue the boiling for five minutes. Pour off the boiling water through a

[22] Abderhalden, Schutzfermente des tierischen Organismus. Berlin, Julius Springer, 1912. Münch. med. Wochen., vol. 59, 1912, pp. 1305, 1939, 2172.

gauze strainer or plaited filter, taking care that the coagulated bits of placenta remain as far as possible in the dish. Pour on more water and boil again for five minutes. Now test a portion of the water for the biuret reaction as follows: To about 10 c.c. of the water, add 5 c.c. of a 33 per cent. caustic soda solution, mix and layer enough of a very dilute (0.25 per cent.) solution of cupric sulphate on top to make a layer 0.25–0.5 cm. deep. A red color at the zone of contact constitutes a positive reaction. If the process has been carried out rapidly, the test will always be negative.

In place of the biuret reaction for peptone the more delicate triketohydrinden hydrate, Hoechst, may be used. This gives a blue color with compounds which have a COOH group and an amino acid in the *a* position. Albumen, peptone, polypeptids and animo acids react. Place 10 c.c. of the fluid in a large test tube, add exactly 0.2 c.c. of a 1 per cent. aqueous solution of triketohydrinden hydrate, bring quickly to boiling and continue the boiling for one minute without interruption. When the reaction is positive, a beautiful violet blue color develops in a short time. When negative, the solution remains colorless, or takes on a yellowish tone. The use of the triketohydrinden hydrate is recommended, but it *must not be used for the test proper unless the placental tissue, as prepared above, has been shown to react negatively with it.*

If the water in which the placenta is cooked gives one or other of these reactions for peptone, the boiling must be continued as before till the reaction is negative. When this occurs pour the last cooking water, together with the placental tissue, into a wide-mouthed flask. Cover with a layer of toluol, and cork tightly. The entire process requires not more than 1/2 to 1 hour, and furnishes material for several hundred tests.

(2) *Extraction shells.* S. & S. 579. These should be kept under water and a layer of toluol, and should be tested for permeability to Witte's peptone before use. After being used, they may be carefully washed in running water and used again. They should never be used dry.

(3) *The patient's serum.* This must not be shaken. It is recommended to allow about 10 c.c. of blood to flow directly into a centrifuge tube. As soon as the clot forms centrifugate, pour off the serum, and use at once for the test. If the serum contains any hemoglobin it cannot be used.

(4) Small beakers, Erlenmeyer flasks or especially designed cylinders of such a size that the shells above mentioned fit into them leaving not more than 0.25 cm. between the shell and the wall.

(5) Reagents for the biuret reaction, or triketohydrinden hydrate, or both.

(6) Incubator at 37° C.

(7) Distilled water.

Technique.—In one of the extraction shells, previously tested as to its permeability, washed, and kept as above described, place about 1 gramme of the placental tissue, crumbling it between the fingers into pieces the size of a wheat grain while doing so. Pour about 2 or 3 c.c. of the patient's serum over the tissue in the bottom of the shell. Add a few drops of the toluol to prevent putrefaction. Now hold the shell at the top, and rinse the outside thoroughly under the tap. Place the shell in one of the beakers, described above, and add 15 c.c. of water, outside the shell. A thin layer of toluol is placed also on the outside. Controls should be prepared in the same way, consisting of the patient's serum alone, and of the placental tissue alone respectively. Place in the incubator for 12–16 hours. Now remove 10 c.c. of the fluid outside of the shell, by means of a pipette thrust below the level of the toluol, and test this for peptone with one or both of the reagents described above. When triketohydrinden hydrate is to be used, it is especially important that as little toluol as possible be transferred to the test tube, as overheating is then apt to occur. A glass rod may be placed in the test tube to prevent bumping.

A positive result is indicated when the dialysate gives a positive reaction for peptone.

The method has been verified by R. Franz [23] and by Frank and Heimann.[24]

The polariscope method of Abderhalden, which is given in detail in the monograph first cited above, is here omitted because it is so complicated as to fall outside the domain of the usual clinical laboratory.

Moro's Alexin Test [25] (Estimation of the Complement Content of a Patient's Serum.)—*Principle.*—The patient's serum, in increasing quantities, is mixed with a fixed quantity of washed erythrocytes, and an amount of *inactivated* (*i.e.*, complement free), immune serum (amboceptor serum), sufficient to cause solution of the red cells. After incubation for 2 hours, the degree of hæmolysis is noted. The amount of the patient's serum required to bring about hæmolysis affords an approximate measure of the complement (alexin) content of the serum.

Apparatus.—(1) A 10 per cent. suspension in physiological salt solution of washed sheep's blood-cells.

(2) Antisheep immune serum, of high potency, and inactivated, to serve as amboceptor.

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[23] Franz, Münch. med. Woch., vol. 59 (1912), p. 1702.

[24] Frank and Heimann, Berl. klin. Woch., vol. 49 (1912), p. 1706.

[25] Münch. med. Woch. (1907), Nos. 21 and 31, and personal communication.

(3) The serum to be tested, *not inactivated.* About 5 drops drawn from the ear will suffice.

(4) Volumetric pipettes, in 5–100's or 1–10's.

(5) Test-tubes, about 5 mm. inside diameter by 5 cm. long.

(6) A Sahli hæmometer.

Technique.—The hæmolytic system is set up as follows: 0.1 c.c. sheep blood suspension, + 0.1 c.c. immune serum diluted 1–500 (titre 1–1000 to 1–1500), + 0.025 c.c. (0.1 c.c. of a 1 in 4 dilution), fresh patient's serum.

Incubation is followed either by complete hæmolysis or by incomplete solution, leaving a residue of red cells.

To measure this residue, the fluid is centrifugated, the supernatant fluid is removed, and the sediment, consisting of red-cells, is dissolved in 0.1 c.c. n/10 HCl. The yellow to brown tint of the resulting solution depends upon the transformation of the hæmoglobin into hæmatin hydrochloride. This fluid is then placed in the graduated tube of the Sahli hæmometer, and its color is compared with that of the standard solution.

Three findings are possible.

(1) *The color may be paler than that of the standard solution.* This may be indicated thus, $n < 0.1$, n representing the amount of fluid in which the red cell sediment must be dissolved, to make it match the shade of the standard solution.

(2) It may be equal; $n = 0.1$.

(3) *It may be darker in color* than the standard solution. In this case the hæmatin solution is diluted until the colors match. Should this happen, for example, on the addition of 0.25 c.c. of water, it is indicated by writing $n = 0.1 + 0.25$, or, for short, 0.35.

Suppose that in the wholly undissolved control, the red cells give a value, thus estimated, of 0.8. Then:

(*a*) In the complete lack of complement in the patient's serum, the resulting value also will be 0.8.

(*b*) Values between 0.75 and 0.4 indicate a more or less markedly reduced complement content.

(*c*) The normal complement content ranges from 0.4 to 0.1.

(*d*) Values between 0.1 and 0.0 indicate a complement content above normal.

The following modification is to be preferred, as it eliminates the occasionally disturbing influence of the native amboceptors for sheep's cells sometimes found in human serum.

0.1 c.c. of a 10 per cent. suspension of washed human red cells, + 0.1 c.c. of an appropriately diluted antihuman immune serum from a rabbit (so diluted that 0.1 c.c. will contain the amount needed to dissolve the 0.1 c.c. red cells), + 0.025 c.c. fresh patient's serum.

The rest of the test is carried out as above.

Antihæmolytic Reactions.

complement deviation, of fixation, tests.

Principle.—Whenever proteid antigens are mixed with their homologous specific antibodies, in the presence of active, (*i.e.,* complement containing), normal serum, fixation of the complement takes place. This may be recognized by the fact that the mixture, in spite of the fact that it contains complement, is no longer able to serve as complement in bringing about lysis in a hæmolytic system, consisting of red blood-cells "sensitized" or charged with a hæmolytic amboceptor. This complement fixation is specific, and permits the recognition of certain antigens, in part of bacterial origin, or of their corresponding antibodies, in the material to be examined; and in many respects constitutes a method comparable to the precipitin reactions.

practical applications.

Demonstration of Proteid Antigens (*e.g.,* determination of human proteid in blood-stains; or of bacterial substances in the serum of patients).

Method of Rickmann.[26]—*Apparatus.*—(*a*) A serum of high potency, immune to the particular antigen to be determined in the material to be tested. Its titre must be known. (For the preparation see p. 22, Precipitin Reactions.)

[26] Arbeit. a. d. K. Inst. f. exp. Ther. in Frankfurt, 1907.

(*b*) *Complement containing serum of a normal guinea pig, obtained fresh the day it is to be used.* The simplest procedure is to stun the animal by a blow at the base of the skull. Then cut its throat and collect the blood in a Petri dish or other suitable receptacle. The serum may be allowed to separate spontaneously over night in the ice-box, or, better, separated at once by centrifugation. Enough serum to furnish complement for several Wassermann tests may be obtained from a guinea pig without killing the animal, by the following method, modified by the *Translator* from that of Zahn:[27] trim the hair from about an ear with scissors. Rub the ear with chloroform or xylol, till the ear becomes hyperæmic. Incise one of the larger veins of the ear with a sharp scalpel, and fit a small distillation flask provided with a side arm as closely as possible over the ear. The side arm of the distillation flask is connected by means of rubber tubing with some form of suction apparatus, such as a Chapman pump. If the flask is held in such a manner that the side arm points upward, the blood will run to the bottom of the flask, as it flows from the incision. About 10 c.c. of blood can be readily collected in this way. This should be poured into a centrifuge tube and centrifugated at once to cause separation of the serum.

[27] Münch. med. Wochenschr., vol. 59 (1912), 861.

The animal may be used repeatedly at intervals of a week or two.

(*c*) *A 5 per cent. suspension of sheep or beef red blood-cells, washed several times with salt solution,* to remove serum. To prepare, dilute the defibrinated blood with salt solution, centrifugate, remove the supernatant clear fluid, and replace with a like amount of fresh salt solution, and again centrifugate. This is repeated at least three times. The washed cells are finally mixed with twenty volumes of salt solution.

(*d*) *A perfectly clear solution* of the material to be examined. (Small blood-stains may be dissolved in the same manner as for the precipitin reaction.)

(*e*) A known solution of the antigen whose presence is to be determined in the material under investigation (*i.e.,* horse serum, for example; or, if we are dealing with the serum of a typhoid patient, then a typhoid bacillus extract).

Lüdke [28] gives the following directions for preparing such an extract: A suspension of a 24-hour-old agar culture is heated for half an hour at 60° to kill the organisms, and then centrifugated. The sediment, consisting of bacteria, is dried, and placed in a sterile agate mortar. It is then ground and at the same time

[28] Zur Kenntnis der Komplemente, Würzburg (1908).

sterile salt solution is added, drop by drop. More salt solution is added after the grinding is completed, to form a slightly opalescent fluid.

Leuchs [29] gives the following directions: Several Kolle's dishes, the surface of each of which about corresponds to 10 agar tubes, are inoculated with the organism desired, and placed in the incubator for 24 hours at 37°. The bacteria are washed off with sterile distilled water. The suspension so obtained is placed at 60° for 24 hours to kill the bacteria, and is then placed in a shaker for 24 hours to bring about autolysis. The suspension is then thoroughly centrifugated, and the clear, supernatant fluid, to which 0.5 per cent. phenol is added, is kept in the ice-box, where it remains unchanged for at least eight weeks.

(*f*) Rabbit's serum of high potency, immune to beef or sheep's blood, inactivated by heating for a half hour at 56° (amboceptor serum). This, like all the fluids used in this test, must be absolutely clear.

(*g*) Test-tubes, 0.9 to 1 cm. diameter by 10 cm. long.

(*h*) Volumetric pipettes, 0.1 c.c. in 1–100's and 1 c.c. and 10 c.c. in 1–10's.

(*i*) Physiological salt solution.

(*j*) An incubator at 37°.

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[29] Berlin. klin. Woch. (1907).

(*k*) A water-bath, for inactivation of the sera.

Technique.—Previous to the test itself, the amboceptor serum, complement serum, and proteid immune serum must be titrated.

Titration of the Amboceptor Serum.—In each of a series of test-tubes are placed 1 c.c. of the beef blood suspension, 0.1 c.c. of complement serum, and increasing quantities of the inactivated amboceptor serum, somewhat as follows:

1–100 dilution of the amboceptor serum, in salt solution.	0.25 c.c. = 0.0025	undiluted serum
do.	0.15 c.c. = 0.0015	undiluted serum
do.	0.10 c.c. = 0.0010	undiluted serum
1–1000 dilution of the amboceptor serum, in salt solution	0.75 c.c. = 0.00075	undiluted serum
do.	0.50 c.c. = 0.00050	undiluted serum
do.	0.35 c.c. = 0.00035	undiluted serum
do.	0.25 c.c. = 0.00025	undiluted serum
do.	0.15 c.c. = 0.00015	undiluted serum
do.	0.10 c.c. = 0.00010	undiluted serum

One tube containing no amboceptor is also set up to serve as a control. The content of all tubes is then brought to a like volume (2.1 to 2.5 c.c.) by the addition of salt solution. After being thoroughly mixed the tubes are placed at 37° for 2 hours. At the end of this time one notes the smallest amount of amboceptor able to cause complete hæmolysis. For the test proper one and one-half to two times this amount is employed,

the amboceptor being diluted so that the required amount will be contained in from 0.1 to 0.2 c.c. of fluid.

Titration of the Complement.—Now place in each of a series of test-tubes the amount of amboceptor thus determined (*i.e.*, 1 unit, which is the minimum amount required to effect lysis), 1 c.c. of the blood-cell suspension, and decreasing amounts of the complement serum. Of the last approximately the following amounts may be used:

Complement serum undiluted . . . 0.1 c.c.
Complement serum diluted 10 times . . . . . 0.75 c.c. = 0.75 undiluted serum
Complement serum diluted 10 times . . . . . 0.50 c.c. = 0.050 undiluted serum
Complement serum diluted 10 times . . . . . 0.35 c.c. = 0.035 undiluted serum
Complement serum diluted 10 times . . . . . 0.25 c.c. = 0.025 undiluted serum
Complement serum diluted 10 times . . . . . 0.15 c.c. = 0.015 undiluted serum
Complement serum diluted 10 times . . . . . 0.10 c.c. = 0.010 undiluted serum
Complement serum diluted 10 times . . . . . 0.0 c.c. = 0.0 undiluted serum

The total content of each tube is again brought to a like volume by the addition of salt solution, the contents mixed, the tubes placed in the incubator for 2 hours, and the result noted. The amount of complement to be used in the test is

one and a half to two times the unit or smallest solvent dose as thus determined.

The hæmolytic system, consisting of 1 c.c. blood-cell suspension and the amounts as above fixed of amboceptor and complement, is thus established. It must be remembered, however, that the amboceptor serum employed must be of such a strength that the minimum effective dose is contained in from 0.001 to 0.0005 c.c. of the undiluted serum (1000 to 2000 units to the c.c.), when 0.1 c.c. of guinea-pig complement is used.

Titration of the Proteid or Bacterial Immune Serum.—This consists in determining in what dilution the serum is capable of hindering hæmolysis in the above-established system when it is mixed with the corresponding antigen, as, for example, with human proteid or the typhoid bacillus extract, as the case may be. The method follows:

Assuming that we are to test for human proteid in a blood-stain, the following three series of tests are set up:

Series *a* contains 0.1 c.c. human serum diluted 1–100.

Series *b* contains 0.1 c.c. human serum diluted 1–1000.

Series *c* contains 0.1 c.c. physiological salt solution.

To all tubes in each series are then added the

proper amount of complement serum as above determined, and decreasing amounts of the anti-human immune serum, somewhat as follows:

Immune serum, undiluted ..... 0.1 c.c.
Immune serum, diluted 1–10... 0.75 c.c. = 0.075 undiluted serum
Immune serum, diluted 1–10... 0.50 c.c. = 0.050 undiluted serum
Immune serum, diluted 1–10... 0.35 c.c. = 0.035 undiluted serum
Immune serum, diluted 1–10... 0.25 c.c. = 0.025 undiluted serum
Immune serum, diluted 1–10... 0.20 c.c. = 0.020 undiluted serum
Immune serum, diluted 1–10... 0.15 c.c. = 0.015 undiluted serum
Immune serum, diluted 1–10... 0.10 c.c. = 0.010 undiluted serum
Immune serum, diluted 1–10... 0.0 c.c. = 0.00 undiluted serum

All the tubes are brought to a like volume, mixed and placed in the incubator for one hour at 37°, to afford an opportunity for complement-fixation to take place. One c.c. of the blood-cell suspension, and the proper amount of amboceptor serum (2 units), are then added to all the tubes, which are then returned to the incubator for 2 hours. Since the control series *c* contains anti-serum, but no antigen (human serum), it must show complete hæmolysis, while the other two series must show more or less marked inhibition of hæmolysis, if the antiserum is active. Since it is necessary, for the forensic determination of human blood or proteid, that the antiserum shall permit the recognition of the same in dilutions up to 1 in 10,000 (*i.e.*, in 1–10 c.c. of a 1–1000 dilution), such an antiserum only can be em-

ployed for the test as shows marked complement deviation (inhibition of hæmolysis) in the series *b*. For the test proper one uses an amount of the antiserum only slightly larger than the minimum inhibiting dose as determined above.

Lüdke employed, in his experiments on the recognition of typhoid bacillus antibody in the blood of patients 0.1 c.c. of his bacillary extract. This corresponds to our series *a* above; one might then, for the series *b*, use 0.01 c.c. of such an extract.

The tests, somewhat elaborate, indeed, preliminary to the test proper are thus completed. As all the fluids employed except the complement can be kept almost indefinitely frozen (the freezing apparatus called "Frigo," of Morgenroth, and sold by the Kny-Scherer Company, of New York, serves admirably for this purpose), a single titration serves for a long time. The titration of the complement only needs to be repeated before each test, and this causes little delay.

The Test Proper.—In each of a series of test-tubes place the following:

(1) The titrated amount of complement serum.

(2) The titrated amount of antihuman (or antibacterial) serum.

(3) Decreasing amounts of the fluid to be tested.

For the forensic determination of blood, one would use about the following amounts of the last:

0.1 c.c. dilution 1–10 = 0.01 c.c.
0.1 c.c. dilution 1–100 = 0.001 c.c.
0.1 c.c. dilution 1–1000 = 0.0001 c.c.
0.1 c.c. dilution 1–10000 = 0.00001 c.c.
0.0 c.c. = 0.0 c.c.

In the determination of antibacterial substances in the blood, somewhat larger amounts, ranging from 1 c.c. downwards to 0.001 c.c., are employed. Tubes without antiserum serve as controls.

The tubes, having been brought to a like volume and mixed, are placed in the incubator for one hour. One c.c. of the blood-cell suspension and 1.5 to 2 units of the corresponding amboceptor are then added. After a second incubation of two hours, all the controls will show hæmolysis, while in the test series proper a more or less marked inhibition of hæmolysis will be observed if the test is positive. The method is fully as specific as the precipitation method, and because of the use of small amounts of antiserum, the danger of heterologous reactions is very slight.

DETERMINATION OF ANTIBODIES.

In the serum, cerebrospinal fluid, or tissues of patients (in typhoid, meningococcus, gonococcus, and staphylococcus infections, in pneumonia, and in syphilis).

Apparatus.—The apparatus needed is the

same as that described on page 75, except [30] instead of (*a*), the perfectly clear serum, or spinal fluid of the patient; and in place of (*d*), a solution of the antigen, the homologous antibody of which is to be determined. In the case of a bacterial infection, therefore, the bacterial extract described under (*e*), page 77 is employed. For the diagnosis of syphilis or a metasyphilitic affection (tabes, general paralysis, etc.) an extract of syphilitic organs is used. (In the first five methods described below, and in method 7, a beef blood hæmolytic system can be substituted for the sheep blood system. According to Peters,[31] this has the advantage that human serum contains no native amboceptor for beef blood-cells, and the clumping and precipitation which is apt to annoy one in the Noguchi antihuman system does not occur.—*Trans.*)

Diagnosis of Syphilis.

Method of Wassermann and Meier (Berlin Method).—Michaelis [32] gives the following instruc-

[30] Finkelstein (Berl. klin. Woch. (1909), No. 35), describes special glassware for use in the complement-fixation test. This consists of a funnel, the stem of which, 40 mm. long by 1 mm. inside diameter, is calibrated. The funnel holds 7 or 8 c.c. After the incubation period has elapsed, the funnel, containing the test, is centrifugated for a few minutes, forcing the undissolved red cells into the calibrated stem. The height of the column of red cells is used as an index of the degree of hæmolysis.

[31] N. Y. Med. Jour., vol. 94 (1911), 21.

[32] Berl. klin. Woch (1907).

tions for preparing the antigen: The liver of a syphilitic fœtus is preserved frozen. A few days before the test is to be made, a small piece is removed. It is first thoroughly ground in a mortar with sea sand. Five parts of physiological salt solution and ½ part 5 per cent. phenol are then added, and the mixture is placed in the shaker for several hours. It is then placed for 4 days or longer in the ice-box (but *not* under 0°). The extract is now ready for use without filtering or centrifugating.

Just before the test, small portions are cleared by centrifugating at high speed, and, when ready for use, present an opalescent appearance, but free of visible floating particles. This fluid contains the syphilis antigen.

Levaditi dries the freshly ground organ *in vacuo* over calcium chloride and sulphuric acid, and pulverizes it in a sterile mortar. One part of the powder is rubbed up with 30 parts of salt solution, as required, placed in the ice-box for 12 hours, and the clear fluid remaining after centrifugating is used.

The extract must meet the following requirements:

(1) 0.2 c.c. must cause complete inhibition of hæmolysis with 0.1 c.c. of known syphilitic serum.

(2) 0.2 c.c. must not cause any inhibition with 0.2 c.c. of known non-syphilitic serum.

(3) 0.5 c.c. must cause no inhibition by itself.

If this occurs it is diluted, but only so far that requirements 1 and 2 are still complied with.

Since many extracts deteriorate rapidly on standing, Seligmann and Blum[33] recommend that the serum of each patient *should be tested not only with one, but with three or four extracts.*

The necessary controls are the serum or spinal fluid of a non-syphilitic individual and of a known syphilitic individual, and an *extract of the liver of a known non-syphilitic infant.*

The titration of the amboceptor, as given on page 79, must precede the test proper.

One next determines the smallest amount of the extract which is able to bind the complement without the addition of immune serum. For this purpose, 1.0, 0.8, 0.6, 0.4, 0.2, 0.1 c.c. respectively of the extract are mixed with 0.1 c.c. complement serum, placed in the incubator for one hour, after which 1 c.c. of 5 per cent. suspension of sheep's cells and the requisite amount of antisheep amboceptor are added. The content of all tubes is brought to 2.5 c.c. by the addition of salt solution. The tubes are then again placed in the incubator, and are inspected at the end of two hours. If the extract inhibits hæmolysis in doses of 0.5 c.c. or less, it must be diluted. (See requirement 3, above.) In the same manner one determines at the same time how large an amount of the extract is required to give complete inhibition with

[33] Berl. klin. Woch. (1909), No. 24.

0.1 c.c. of known syphilitic serum, so as to determine whether requirement 1 is met. If this is the case, the test proper may be begun.

The method of Wassermann [34] and his collaborators is as follows: The serum or spinal fluid of the patient is mixed with complement serum and liver extract, and placed in the incubator for one hour. One c.c. of the sheep's blood suspension and the previously titrated amount of amboceptor serum (see page 79) are then added, the volume brought to 2.5 c.c. with salt solution, and the tubes returned to the incubator for two hours, after which the result is noted. The amounts to be used of extract and serum or spinal fluid are given in the table below, which reproduces an experiment of Wassermann and Meier. The necessary controls are also shown in the table.

Blood serum, inactivated, or spinal fluid not inactivated.	Organ extract.	Complement.	Result.
Patient's 0.2.....	Luetic 0.2	0.1 (It is undoubtedly better to use 1½–2 units of a titrated complement. Cf. p. 80 *Trans.*)	Inhibition
Patient's 0.2.....	Normal 0.2	0.1	Hæmolysis
Patient's 0.2.....	None	0.1	Hæmolysis
Patient's 0.4.....	None	0.1	Hæmolysis
Known luetic 0.2.....	Luetic 0.2	0.1	Inhibition
Luetic 0.2.....	Normal 0.2	0.1	Hæmolysis
Non-luetic 0.2.....	Luetic 0.2	0.1	Hæmolysis
None.....	Luetic 0.2	0.1	Hæmolysis
None.....	Luetic 0.4	0.1	Hæmolysis
None.....	None	0.1	Hæmolysis
None.....	None	0.1	Hæmolysis

[34] Wassermann, Neisser, Bruck, and Schucht, Zeitschr. f. Hyg., vol. 45 (1906); Wassermann and Meier, Deutsch. med. Woch. (1907).

(Neue, *Münch. med. Woch.*, vol. 59 (1912), 121, insists that larger amounts of spinal fluid than those specified above, up to even 1.0 c.c., should be used.—*Trans.*)

When the reaction is positive, the phenomenon of complement deviation (inhibition of hæmolysis) will be found only in the known luetic control and in the patient's serum, while all the other controls show complete hæmolysis.

Wechselmann[35] recommends the removal by absorption of the "complementoids," because of the fact that certain luetic sera react negatively through the phenomenon of "complementoid stoppage." For this purpose 0.9 c.c. of the inactivated patient's serum is shaken up with 3 c.c. of physiological salt solution and 0.6 c.c. of an approximately 7 per cent. suspension of freshly precipitated barium sulphate. After standing for one hour at 37°, the mixture is clarified by centrifugation. One to 2 c.c. of the centrifugate, representing 0.2 to 0.4 c.c. of the serum, is used for the test.

Method of Taege[36] **(Breslau Method).**—(1*a*) Taege prepares the organ extract as follows: The liver of a syphilitic fœtus is chopped fine or ground in a meat-grinder. The pulp is weighed and mixed in a flask with four times its weight of

[35] Zeitschr. f. Immunitätsforch. vol. 3 (1909).

[36] Münch. med. Woch. (1908), No. 33.

absolute alcohol. The flask is allowed to stand at room temperature for 24 hours, or is placed in the shaker over night. It is then filtered, the filtrate is placed in a shallow dish, and evaporated to a paste *in vacuo* at 40° and 60 mm. pressure of mercury. One gramme of the paste is ground to a fine suspension in 100 c.c. of 0.85 per cent. sodium chloride solution, shaken for 24 hours in the shaker, and preserved in the ice-box. A milky fluid results. Its keeping qualities may be increased by adding 0.3 per cent. phenol.

(1 *b*) The concentration of the extract *in vacuo* may be avoided by mixing 1 part of the fresh ground liver with 10 parts of absolute alcohol, and shaking for 24 hours with glass beads. The filtrate is the stock solution.

For use, 1 part of this extract is mixed with 3 parts of salt solution.

(2) *The patient's serum* inactivated, as well as the known syphilitic and non-syphilitic sera, are diluted with 4 parts of physiological salt solution.

(3) The *complement serum* is diluted with 9 parts of salt solution.

(4) The amboceptor serum is preserved by evaporating it to a dry powder *in vacuo,* and sealing in small brown glass vials containing 0.3 gramme each. Dissolve the content of one vial in 3 c.c. of distilled water, and mix 0.1 c.c. with 60 c.c. of salt solution for use.

The *hæmolytic system* is set up as follows: In each of 6 small test-tubes, numbered serially, is placed 2 c.c. of salt solution. Then add:

Tube 1–1 c.c. amboceptor.
2–0.5 c.c. amboceptor + 0.5 c.c. salt solution.
3–1 c.c. amboceptor diluted 1 to 2 (1 c.c. amboceptor + 2 c.c. salt solution).
4–1 c.c. amboceptor diluted 1 to 3 (1 c.c. amboceptor + 3 c.c. salt solution).
5–1 c.c. amboceptor diluted 1 to 5 (1 c.c. dilution 1 to 2 + 1 c.c. salt solution).
6–1 c.c. amboceptor diluted 1 to 7 (1 c.c. dilution 1 to 3 + 1 c.c. salt solution).

One c.c. complement and 1 c.c. sheep's blood-cell suspension are then added to all tubes, the tubes shaken, and placed in the incubator.

Tube 1 should show complete hæmolysis in 15 minutes.

Tube 2 should show complete hæmolysis in 30 minutes.

Tubes 3 and 4 should show complete hæmolysis in from 30 to 45 minutes.

Tubes 5 and 6 should show complete hæmolysis in two hours.

If hæmolysis occurs within a shorter time, the amboceptor should be diluted with an equal volume of salt solution. If it takes place more slowly, twice as much of the stock amboceptor solution should be used in making the dilution for use (0.2 c.c. instead of 0.1).

To test the antigen, mix 2 c.c. with the hæmo-

lytic system (1 c.c. each amboceptor, complement and blood suspension). Complete hæmolysis must occur after incubating for one hour.

A source of error, which may possibly disturb the result, lies in the fact that in rare cases a *normal* serum may cause inhibition of hæmolysis with an otherwise trustworthy antigen. This error may be avoided by using an antigen only after one has determined that it causes no inhibition with 10 or 12 different known normal sera. If the antigen is weak, and produces no marked inhibition with known luetic sera, special tests must be made to determine in what amounts it should be used to obtain the desired result. Finally, it is a good plan to keep on hand as large a stock of known luetic sera as possible, since the luetic sera lose their power to react when kept very long.

The *test proper* is made as follows:

Tube.	Complement.
1	1 c.c. + 2 c.c. salt solution.
2	1 c.c. + 1 c.c. antigen + 1 c.c. normal serum.
3	1 c.c. + 1 c.c. antigen + 1 c.c. known luetic serum.
4	1 c.c. + 2 c.c. antigen + 0 c.c. serum.
5	1 c.c. + 1 c.c. antigen + 1 c.c. patient's serum.
6	1 c.c. + 0.5 c.c. antigen + 0.5 c.c. patient's serum + 1 c.c. salt solution.
7	1 c.c. + 0 c.c. antigen + 2 c.c. patient's serum.

After remaining for one hour in the incubator, 1 c.c. amboceptor and 1 c.c. sheep's blood suspension are added to all tubes, and they are returned

to the incubator for another hour. Tubes 1, 2, 4, and 7 will then show complete solution, while tubes 3, 5, and perhaps 6, will show no hæmolysis when the reaction is positive.

Method of Hoehne [37] (Frankfurt Method).—An alcoholic extract of the liver of a syphilitic fœtus or infant (1 gramme of liver to 5 c.c. of alcohol) is used as the antigen. The extract is diluted with 3 volumes of salt solution, made fresh for each occasion. It is used in decreasing amounts, as 0.75, 0.5, 0.25 c.c., to which is added 1 c.c. of a 10 per cent. solution of the inactivated patient's serum, while a fourth tube contains the serum only, with no extract. To all tubes is then added 0.5 c.c. of a 20 per cent. solution of guinea-pig serum. The tubes are brought to a like volume (2.5 c.c.) by adding salt solution. One thus obtains the following scheme:

(*A*) *The Test Proper.*—Tube 1—0.75 c.c. extract + 1 c.c. 10 per cent. patient's serum + 0.5 c.c. 20 per cent. guinea-pig serum + 0.25 c.c. salt solution.

Tube 2—0.5 c.c. extract + 1 c.c. 10 per cent. patient's serum + 0.5 c.c. 20 per cent. guinea-pig serum + 0.5 c.c. salt solution.

Tube 3—0.25 c.c. extract + 1 c.c. 10 per cent. patient's serum + 0.5 c.c. 20 per cent guinea-pig serum + 0.75 c.c. salt solution.

Tube 4—0 c.c. extract + 1 c.c. 10 per cent. patient's serum + 0.5 c.c. 20 per cent. guinea-pig serum + 1 c.c. salt solution.

(*B*) *Controls.*—Tube 5—2 c.c. 10 per cent. patient's serum + 0.5 c.c. 20 per cent. guinea-pig serum.

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[37] Berl. klin. Woch. (1909), No. 38.

Tube 6—1.5 c.c. extract + 0.5 c.c. 20 per cent. guinea-pig serum + 0.5 c.c. salt solution.

Tube 7—1 c.c. extract + 0.5 c.c. 20 per cent. guinea-pig serum + 1 c.c. salt solution.

Tube 8—0.75 c.c. extract + 0.5 c.c. 20 per cent. guinea-pig serum + 1.25 c.c. salt solution.

Tube 9—0.5 c.c. extract + 0.5 c.c. 20 per cent. guinea-pig serum + 1.5 c.c. salt solution.

(*C*) *Controls.*—Known luetic and known non-luetic sera are set up as under *A*.

The tubes are then placed in the incubator at 37° for 1¼ hours, after which are added 0.5 c.c. of a dilution of the amboceptor, *freshly prepared for the day,* and so made that the amount mentioned will contain from 2½ to 3 hæmolytic units and 1 c.c. of a 5 per cent. sheep's blood suspension. The tubes are then returned to the incubator for two hours.

When the reaction is positive, tubes 1, 2, and 3 will show a more or less marked inhibition of hæmolysis, and tubes 4 to 9 will show complete hæmolysis.

Simplified and Improved Method of Stern[38] **(Breslau Method).**—*Apparatus.*—1. Extract of syphilitic liver, as described on p. 90, under (1 *b*), but prepared in the proportion of 1 part of liver to 9 parts of absolute alcohol. For use the extract is diluted with 9 parts of salt solution.

2. 2.5 per cent. suspension of washed sheep blood-cells.

[38] Zeitschr. f. Immunitätsforch., vol. I, No. 3.

3. The patient's serum *not inactivated.* This serves also as complement, so that the use of guinea-pig serum is unnecessary. Serum sent in from a distance, and as much as 48 hours old, and even blood from a cadaver, can be tested under favorable conditions.

4. Antisheep amboceptor serum. For use it is diluted so that 1 c.c. contains 5 to 6 units for 1 c.c. of the 2.5 per cent. sheep's blood-cells.

5. Known normal and known syphilitic sera, *in the active* condition, to serve as controls.

Technique.—Tube 1—0.2 c.c. patient's serum + 1 c.c. 10 per cent. extract.

Tube 2—0.2 c.c. patient's serum + 0.5 c.c. 10 per cent. extract.
Tube 3—0.2 c.c. patient's serum + 1 c.c. salt solution.
Tube 4—0.2 c.c. normal serum + 1 c.c. 10 per cent. extract.
Tube 5—0.2 c.c. normal serum + 0.5 c.c. 10 per cent. extract.
Tube 6—0.2 c.c. normal serum + 1 c.c. salt solution.
Tube 7—0.2 c.c. luetic serum + 1 c.c. 10 per cent. extract.
Tube 8—0.2 c.c. luetic serum + 0.5 c.c. 10 per cent. extract.
Tube 9—0.2 c.c. luetic serum + 1 c.c. salt solution.
Tube 10—1 c.c. salt solution + 1 c.c. 10 per cent. extract.

All tubes are brought to a volume of 2 c.c. by adding salt solution, and are placed at 37° for one hour. Then add to each tube 1 c.c. 2.5 per cent. sheep's blood suspension and 1 c.c. amboceptor solution, and return tubes to the incubator for ¼ to ½ hour, when it will be found that most of the controls already show hæmolysis. The tubes are then allowed to stand for 1 to 1.5 hours at room temperature, when the final reading is taken.

Tubes which become completely or markedly

hæmolyzed are to be regarded as negative; moderate and delayed hæmolysis, with a large sediment of intact red cells, as positive.

When the reaction is positive, tubes 1, 2, 7, and 8 show inhibition; tubes 3, 4, 5, 6, 9, and 10 show complete solution of the red blood-cells.

Experience with this method, which is still somewhat limited, is, however, most favorable. It seems to be *considerably more delicate* than the original Wassermann method. *Non-specific* inhibition has, however, been observed in cachectic patients.

Simplified Method of Bauer.[39]—*Apparatus.*—(1) Fresh guinea-pig serum, diluted to 10 per cent. with salt solution, as complement.

(2) Organ extract. The liver of a syphilitic fœtus or infant is ground up and shaken over night with 10 volumes of absolute alcohol. The clear centrifugate serves as the stock solution and keeps indefinitely. For use it is diluted with three parts of salt solution. If 1 c.c. inhibits the hæmolysis of 1 c.c. of 5 per cent. sheep's cells, in the presence of 0.2 c.c. of normal human serum and 0.1 c.c. of complement serum, a higher dilution is to be used.

(3) Five per cent. suspension of washed sheep's blood-cells.

(4) The patient's serum, heated at 56° for ½ hour.

[39] Deutsch. med. Woch. (1908), No. 16.

(5) Known luetic and known normal serum, similarly inactivated.

The rest of the apparatus is that described under *g-k,* p. 78. Since the normal (native) amboceptor for sheep's cells present in the human serum tested serves as the hæmolytic agent, a specific immune amboceptor is not required.

Technique.—Tube 1—0.2 c.c. patient's serum + 1.0 c.c. extract + 1 c.c. 10 per cent. guinea-pig serum.

Tube 2—0.2 c.c. patient's serum + 1.0 c.c. salt solution + 1 c.c. 10 per cent. guinea-pig serum.

Tube 3—0.2 c.c. normal serum + 1.0 c.c. extract + 1 c.c. 10 per cent. guinea-pig serum.

Tube 4—0.2 c.c. normal serum + 1.0 c.c. salt solution + 1 c.c. 10 per cent. guinea-pig serum.

Tube 5—0.2 c.c. luetic serum + 1.0 c.c. extract + 1 c.c. 10 per cent. guinea-pig serum.

Tube 6—0.2 c.c. luetic serum + 1.0 c.c. salt solution + 1 c.c. 10 per cent. guinea-pig serum.

The tubes are placed in the incubator for one-half hour, and 1 c.c. sheep's blood suspension is then added. The tubes are then returned to the incubator for one-half to one hour. Tubes 1 and 5 should show complete inhibition of hæmolysis, and tubes 2, 3, 4, and 6 complete solution. Only complete inhibition is to be regarded as indicating a positive reaction.

If considerable amounts of serum are available, several tests should be made, using varying amounts of the latter. In nurslings and young children, and in about 10 per cent. of adults, the

reaction miscarries, because the normal amboceptor against sheep's blood is lacking. This defect may be corrected by adding to such sera a like amount of normal human serum, known to contain the amboceptor. As lipæmic serum cannot be used, it is better to draw the blood for the test when the patient is fasting. Numerous objections against Bauer's method have recently been offered, but future experience will have to determine whether these are just.

Simplified Method of Tschernoguboff.[40]—*Apparatus.*—1. Antihuman hæmolytic immune serum, derived from the rabbit (amboceptor serum).

2. Extract of a syphilitic liver, as antigen. The ground-up liver of a congenitally syphilitic fœtus is dried and powdered. When needed, 0.5 gramme of this powder is extracted for 12 to 15 hours in the ice-box with 25 c.c. of 95 per cent. alcohol. The extract is diluted with 50 to 200 volumes of salt solution for use.

3. The patient's blood. This serves at once as the complement, red cell emulsion, and as antisyphilitic antibody. It is obtained as follows: Prick the patient's finger, and with a pipette, or Zeiss blood-counting pipette for white cells, draw up 0.1 c.c. of blood. This is repeated four times, the blood obtained being each time mixed with salt solution or liver extract, and placed in small test-

[40] Berlin klin. Woch., (1908), No. 47.

tubes. In tubes 1 and 2 we have 0.1 c.c. blood + 1.0 c.c. salt solution, and in tubes 3 and 4, 0.1 c.c. blood + 1.0 c.c. dilute liver extract.

The rest of the equipment is as described on page 78.

Technique.—The four tubes above mentioned are placed for one hour in the incubator, after which 0.25 c.c. amboceptor serum is added to tubes 1 and 3, and a like amount of salt solution to tubes 2 and 4. The tubes are then returned to the incubator for two hours, and are then placed in the ice-box. The result is read the following morning. Tube 1, which contains no antigen, should show hæmolysis, and tube 2, which contains no amboceptor, should show none. If this is the case, and if tube 3, which contains the test proper, shows inhibition and tube 4, which is devoid of amboceptor, also shows no hæmolysis, the result is positive.

The various methods of carrying out the complement deviation test described above have in part already justified themselves to an extraordinary degree. It has, however, been strikingly demonstrated that the reaction also takes place with *normal* liver extract, though, indeed, less intensely, as well as with an extract of guinea-pig's heart muscle; so that, in spite of its practical value, the theoretic basis on which it rests has become doubtful.

Method of Landsteiner, R. Müller, and Pötzl.[41]— According to these writers, the diagnosis of syphilis may be simplified as follows: Instead of the extract of luetic tissue, the following fluid is used: 1 gramme of the heart muscle of a guinea-pig is cut up with scissors, and then ground in a mortar with a small amount of quartz sand. The finely divided, pasty material is placed in a flask and thoroughly shaken with 50 c.c. of 95 per cent. alcohol. It is then digested for two hours at 60°, during which time it is occasionally shaken. It is then filtered through paper, and the filtrate kept at room temperature.

For the rest, the same equipment is required as above, except that Müller uses a 50 per cent. emulsion of sheep's blood instead of a 5 per cent. emulsion.

Technique.—In each of a series of test-tubes place 10 drops of salt solution and 1 drop of complement serum; then add to

Tube 1, 1 drop of the patient's serum, inactivated.

Tube 2, 1 drop of the patient's serum, inactivated + 2 drops extract.

Tube 3, 1 drop known luetic serum, inactivated.

Tube 4, 1 drop known luetic serum, inactivated + 2 drops extract.

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[41] Wien. klin. Woch., 1907, and Müller, *ibid.*, 1908.

Tube 5, 1 drop known normal serum, inactivated.

Tube 6, 1 drop known normal serum, inactivated + 2 drops extract.

Tube 7, 2 drops extract.

After remaining at 37° for one hour, 1 drop of the blood suspension and double the amount of amboceptor required to effect hæmolysis are added. The tubes are returned to the incubator for 1½ hours, when the result is read.

Cancer Diagnosis.

Method of von Dungern.[42]—*Apparatus.*—(*a*) *Antigen Solution.*—This is prepared in one of the two following ways: 1. The tumor tissue, freed as much as possible of fat and degenerated portions, is ground fine, and mixed with twenty volumes of acetone. Extract for one or two weeks at room temperature, with occasional shaking. Filter. For use, evaporate a portion of the acetone solution to dryness at 37°, and take up with half the amount of absolute alcohol. Dilute this with salt solution according to the results of the titration, which is carried out as in the case of the alcoholic syphilis antigens. Of three antigens, Von Dungern used one diluted 1–40, and the others diluted 1–10. The largest amount, twice which is not of itself anticomplementary, is to be used

[42] Münch. med. Woch., vol. 59 (1912), 65, 1098, 2854.

for the test. 2. Receive 10 c.c. human blood (preferably obtained from a patient suffering from general paralysis) in a container in which has been placed 0.1 c.c. of a 20 per cent. solution of sodium oxalate. Wash the cells three times with physiological salt solution, and after the last washing centrifugate strongly to produce as thick an emulsion as possible. Weigh this and to one part by weight of cells, add twenty parts by volume of C. P. acetone. Allow this to stand for three days at room temperature, with occasional shaking. Filter, evaporate the filtrate to dryness in the incubator, and take up the residue with enough 96 per cent. alcohol to form a 1 per cent. solution. Usually this requires about ten times as much alcohol as was used of the acetone. The extract is completely soluble in the alcohol if the latter is added as soon as the acetone has evaporated. For use, the alcoholic solution is slowly mixed with four parts of physiological salt solution, and thoroughly shaken. 0.8 c.c. is the usual dose for the reaction, but this should of course be fixed by titration. The salt solution should be made with C. P. sodium chloride, Kahlbaum.

Von Dungern prefers the extract of blood-cells to that made from cancer tissue.

(*b*) The *serum to be tested,* and when available, positive and negative controls. Draw the blood from the vein aseptically and, as soon as clotting takes place, remove the serum and preserve in a

sterile container for one or two days in the ice-box. Then add two volumes of n/50 caustic soda solution and inactivate the mixture for one-half hour at 54° (*not* 56°). The n/50 caustic soda solution is prepared by diluting n/10 caustic soda with four volumes of physiological salt solution. The stock solution of caustic soda must be accurately prepared from chemically pure materials; it must be free from carbonate, and must be kept protected from contact with the air.

(*c*) Fresh guinea pig serum, diluted 1–20. 1 c.c. is used.

(*d*) Five per cent. emulsion of washed beef (or sheep, *Trans.*) cells and the homologous immune serum. The unit of amboceptor is the amount which completely hæmolyses 1 c.c. of the blood cells in 2 hours in the incubator, in the presence of 1 c.c. of the diluted complement serum. Use 2 units for the test.

(*e*) Test-tubes, pipettes, salt solution, etc., as for other tests of the sort.

Technique.—In a series of tubes place 0.6, 0.3, 0.15, and 0.075 c.c., respectively, of the serum-caustic soda mixture above described. To each tube add 1 c.c. of the diluted complement serum and the proper amount of antigen, as determined by preliminary tests. Bring all tubes to a like volume, mix thoroughly, and allow to stand for three hours at room temperature. Then add to each tube 1 c.c. blood suspension and 2 units of

amboceptor. The result is read off after three hours, again at room temperature, and without shaking. Similar tests should be made with positive and negative control sera, when these can be obtained. One control tube in each set should contain 0.2 c.c. serum and 1 c.c. complement, but no antigen.

The above form of the test appeared in the last report above cited, under date of December 24, 1912. With this technique Von Dungern has obtained over 90 per cent. positive reactions in known cases of cancer. Rarely syphilis and surgical tuberculosis have given positive reactions. Von Dungern's experience now embraces 422 cases. The translator obtained highly satisfactory results in a series of about 30 cases, employing the technique recommended in the earlier reports above cited. Reports from other observers are somewhat contradictory, due perhaps to variations in the technique employed.

Diagnosis of Gonorrhœa (Method of Schwartz and McNeil).[43]—*Apparatus.*—(*a*) *Antigen:* Slants of several strains of the gonococcus (9 to 12 were used by S. and McN.), grown for 24 to 48 hours on Tholman's medium containing 2 per cent. of glucose, are washed off with sterile 0.85 per cent. salt solution, 2 c.c. for each tube. The bacteria

[43] Am. Jour. Med. Sc., vol. 141 (1911), 693.

may be scraped from the surface of the medium with a platinum loop. Mix the several strains, pour the mixture into a sterile bottle, and heat in a water-bath at 56° for 30 minutes. Then either (1) place in a shaking apparatus and shake for 24 hours, centrifugate, decant the supernatant fluid, and place the latter in a tightly corked, sterile bottle, add a capillary drop of lysol and preserve in the ice-box; or (2) allow the original suspension to stand in the incubator for several hours, at 37°, instead of shaking. Complete the preparation as above.[44]

The dose of the antigen to be used in the test is an amount which completely inhibits hæmolysis of a fixed amount of red-cell suspension in the presence of a fixed amount of positive serum and complement, provided that twice this amount is not anticomplementary.

The rest of the apparatus is identical with that used for the Wassermann test, page 76, *et seq.* The hæmolytic system may be either a sheep cell system, or a human cell system, as in the Noguchi method.

Technique.—The antigen is titrated against a known positive serum or against the antigonococcic serum of Torrey, as prepared by Parke, Davis & Co.

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[44] This antigen, ready for use, may be obtained from Parke, Davis & Co.

The titration of the complement and amboceptor is carried out as already described on pages 79 and 80, using, however, 0.05 c.c. of a 5 per cent. suspension of sheep's cells instead of 1.0 c.c. For the test proper the customary excess of complement and amboceptor is employed.

The test and necessary controls are shown in the following table:

Patient's serum, inactivated at 56°.	Antigen.	Complement, 10 per cent.	Amboceptor.	Sheep's cells, 5 per cent.	Hæmolysis positive case.
0.15 c.c.	0	0.05	0.05	0.05	Complete
0.10 c.c.	0	0.05	0.05	0.05	Complete
0.05 c.c.	0	0.05	0.05	0.05	Complete
0	double titrated quantity	0.05	0.05	0.05	Complete
0	0	0.05	0.05	0.05	Complete
0	0	0	0.05	0.05	None
0	0	0.05	0	0.05	None
0.15	titrated quantity	0.05	0.05	0.05	None
			0.05	0.05	None
0.10	Same	0.05	0.05	0.05	None
0.05	Same	0.05	0.05	0.05	None
Known positive					
0.10	Same	0.05	0.05	0.05	None
0.10	0	0.05	0.05	0.05	Complete

Place in the incubator at 37° for 2 hours and add

The test is negative in the acute stages of the disease, but is recommended as an aid to differential diagnosis between the chronic conditions due to the gonococcus and similar conditions due to other causes. It is not yet proved that a positive reaction indicates the presence of an active focus

of gonorrhœal infection, but this is probably the case. (S. and McN.)

Schwartz[45] states that subsequent tests have fully confirmed the early favorable impression of the test.

Diagnosis of Tuberculosis (Method of Hammer).[46]—*Apparatus.*—Antigen: This is a mixture of old tuberculin and an extract of tuberculous tissue. It is important that tissue consisting of the tubercular lesion alone should be used so far as possible. Hammer prepared his extract from the tubercular granulations in the capsule of a tubercular knee-joint. It may be extracted in either of the two following ways: (*a*) Cover the tissue with 4 parts of alcohol and extract for three to five days. Filter and dilute for use with 3 parts of physiological salt solution. 0.4, 0.2, and 0.1 c.c. of this dilution are tested against 0.1 c.c. known tubercular serum. For the test proper use the largest amount, twice which amount is not in itself anticomplementary. Or (*b*), the tubercular tissue may be covered with 9 parts of acetone and extracted for 10 days. Filter and evaporate to dryness at 37°. The dry residue of 10 c.c. of the acetone extract is dissolved in 10 c.c. of absolute alcohol, and diluted for use with 10 parts of physiological salt solution. It is titrated as above.

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[45] Personal communication.

[46] Münch. med. Wochenschr., 59 (1912), 1750.

Mixtures of old tuberculin and extract are then prepared as follows:

0.04 c.c. tuberculin + 0.4 c.c. extract.
0.02 c.c. tuberculin + 0.2 c.c. extract.
0.01 c.c. tuberculin + 0.1 c.c. extract.
0.005 c.c. tuberculin + 0.05 c.c. extract.

Of these the largest amount is used, twice which amount is not in itself anticomplementary. The extract alone and the tuberculin alone each reacts with a certain proportion of the cases. The largest percentage of positive reactions is given by a mixture as above.

The other apparatus required is the same as that described under the von Dungern test for carcinoma.

Technique.—The complement and amboceptor are titrated as for the von Dungern test. For the test proper, place in the several tubes arranged for the patient's serum and the customary controls 1 c.c. of the diluted complement. To the front row add the titrated amount of antigen. Mix thoroughly, and then add 1–10 c.c. of the several sera inactivated for half an hour at 56°. This mixture is allowed to stand for three hours at room temperature, after which 1 c.c. of 5 per cent. suspension of beef (or sheep, *Translator*) cells and 2 units of the corresponding amboceptor are added. The reading is made after one hour in the incubator and when all the controls are completely dissolved.

Hammer's article is merely a preliminary contribution. His series consisted of 46 cases of known tuberculosis, of which 97.8 per cent. gave a positive result. The one case which failed to react was possibly cured.

Cobra Venom Reactions.

Inhibition of Cobra Venom Hæmolysis (Psychoreaction of Much and Holzmann).[47]—*Principle.*—In many psychoses the serum of the patient has the ability to neutralize the hæmolytic power of cobra toxin. The clinical significance of the reaction has not yet been determined.

Apparatus.—(*a*) A solution of cobra venom. Dissolve 0.2 gramme toxin in 10 c.c. of distilled water and add 10 c.c. of glycerin. Keep this stock solution in the ice-box.

(*b*) A freshly prepared 10 per cent. suspension of washed human red blood-corpuscles (defibrinated placental blood).

(*c*) The patient's serum, inactivated by heating for one-half hour at 56°. 1.5 to 2 c.c. are required.

(*d*) Small test-tubes, 6 or 7 mm. in diameter.

(*e*) Pipettes, 1 c.c. in 1–10's.

(*f*) Physiological salt solution.

(*g*) An incubator, at 37°.

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[47] Münch. med. Woch. (1909), No. 20, and Omorokow, Berl. klin. Woch. (1909), No. 41.

Technique.—The venom solution for the test is prepared by diluting the stock solution with 50 volumes of salt solution, *e.g.*, 0.2 c.c. toxin to 9.8 c.c. salt solution. In each of a series of tubes place 0.35 c.c. patient's serum and decreasing amounts of venom, 1.0, 0.5, 0.25, and 0.1, respectively. Then add to each tube 0.5 c.c. of the blood-cell suspension. The total content of all tubes is then made 1.85 c.c. by the addition of salt solution. A like series of tubes, with the patient's serum omitted, serves as a control. One thus obtains:

Tube.	Serum.	Cobra toxin.	Blood.	Salt solution.
1	0.35	1.0	0.5	0
2	0.35	0.5	0.5	0.5
3	0.35	0.25	0.5	0.75
4	0.35	0.1	0.5	0.9
5	0	1.0	0.5	0.35
6	0	0.5	0.5	0.85
7	0	0.25	0.5	1.1
8	0	0.1	0.5	1.25

Mix thoroughly, and place the tubes in the incubator for two hours, then in the ice-box for 22 hours more. Before the final inspection, all tubes should be shaken thoroughly. When the reaction is positive, the tube containing the patient's serum, and the smallest amount of toxin able to produce hæmolysis in the absence of serum, remains non-transparent, *i.e.*, shows inhibition of hæmolysis. In the above table, for example, if solution occurs in tubes 5, 6, and 7, but is absent

in 8, the minimum dissolving dose of toxin is 0.25, and tube 3, which also contains this amount of toxin, must show inhibition.

Cobra Venom Reaction of Weil.[48]—*Principle.*—Human blood-cells show varying degrees of resistance to the hæmolyzing power of cobra venom. In syphilis this resistance is increased more than in any other condition. In the earliest stages of the disease the cells may be hypersensitive.

Apparatus.—(1) A solution of cobra venom. The stock solution is a 0.5 per cent. solution, made very accurately with 0.9 per cent. salt solution. It deteriorates rapidly unless kept frozen. For use, dilutions of 1–10,000, 1–20,000, 1–30,000, and 1–40,000 are prepared and kept constantly at a low temperature.

(2) The blood-cells to be tested. Draw the blood from one of the veins at the elbow and discharge about 2 c.c. into 2 per cent. sodium citrate in 0.9 per cent. sodium chloride. The latter must be prepared fresh, or, at most, the day before, and kept on ice. *The mixture of blood and citrate solution must not be shaken.* The cells must be washed at least four times in 0.9 per cent. salt solution. The washing is done in accurately calibrated tubes, and the last washing always at the same speed and for the same length of time. Ac-

[48] Infec. Dis., vol. 6 (1909), 688, and Schwartz, N. Y. Med. J., 95 (1912), 23.

curate dilution of the red cells is very important. The cells are then made up to a 4 per cent. suspension with salt solution, which has been kept on ice if the cells are to be tested the same day. The suspension is to be placed on ice for a short time before the test is made.

Technique.—As the dry venom does not vary, a single preliminary titration against known normal and known syphilitic serum serves for all.

Mix 1 c.c. of the cells with 1 c.c. of each of the venom dilutions. Incubate at 37° for one hour. Mix thoroughly, and place in the ice-box over night. In the morning again mix thoroughly, and make the final reading an hour later. The venom used by Weil titrated as follows:

Non-hæmolysis at 1–10,000 = strongly positive.
Moderate hæmolysis at 1–20,000 = positive.
Partial hæmolysis at 1–30,000 = negative.
Complete hæmolysis at 1–40,000 = hypersensitiveness.

Fresh blood-clot may be broken up in the citrate solution, prepared as above, and tested, using the venom twice as strong. There are three criteria which aid in determining the result, viz., the rapidity with which the hæmolysis begins, the degree of completeness at the time of the final reading, and the tendency of the red cells to sink rapidly to the bottom. These conditions are all reversed in positive tests.

The method is said to have the following advantages over the Wassermann method: It is

simpler and less laborious; a smaller amount of blood is required; scarlatina and leprosy do not react positively; jaundice does not interfere with the test; it is more sensitive than the Wassermann in the late, untreated, and dormant cases in which diagnosis is especially apt to be difficult; it is less quickly abolished by treatment, affording a further diagnostic and therapeutic test.

Cobra Venom Reaction of Kraus, Graff, and Ranzi.[49]—*Principle.*—In certain conditions human blood-serum possesses the ability to activate the hæmolysis of horse's red blood-cells by cobra venom.

Applications.—About 70 per cent. of the cancer sera react positively, and the reaction may remain positive for years after operation, in the absence of any indication of recurrence. It is invariably strongly positive in pregnancy, beginning with the fourth month, and disappearing about three weeks after parturition. Icterus and advanced tuberculosis may also react positively. (Graff and Zubrzycki.[50])

Apparatus.—(*a*) Solution of cobra venom. The stock solution is 1 per cent. in equal parts of glycerin and distilled water. *It should stand several days before it is used,* and keeps extraordi-

[49] Wien. klin. Woch. (1911), No. 28.

[50] Münch. med. Woch., vol. 59 (1912), 574.

narily well in the ice-box. A dilution of 1–5000 is made from this just before use.

(*b*) Patient's serum. Inactivate for 20 minutes at 50°.

(*c*) Horse's red blood-cells, washed three or four times with salt solution and made up to a 10 per cent. suspension in salt solution. Blood a week old can be used after recentrifugating to remove cells spontaneously hæmolyzed.

(*d*) Test-tubes of the ordinary size.

(*e*) Salt solution.

(*f*) Volumetric pipettes, 1 c.c. in 1–100's and 10 c.c. in 1–10's.

(*g*) Incubator at 37°.

Technique.—In two series of four tubes each, place 0.1, 0.2, 0.3, and 0.5 c.c. of the inactivated patient's serum, and fill all tubes to 0.5 c.c. To the first series add 0.3 c.c. of the cobra venom solution, 1–5000, and to the second series 0.15 of the same. Finally, add to all tubes 5 drops of the 10 per cent. horse blood suspension. Shake vigorously, and place in the incubator at 37°. Inspect at the end of 15 and 30 minutes, and after one, two, and three hours. The final inspection determines the result, which Graff and Zubrzycki indicate as follows:

$\varnothing$ = no solution, + = trace of solution, + + = partial solution, + + + = complete or almost complete solution.

Antifermentative Reactions.

estimation of the antitrypsin content of the blood.

Principle.—One determines what amount of trypsin is neutralized, as to its digestive power, by a certain amount of serum or blood. The substance to be digested may be either Loeffler's blood-serum, as ordinarily used by bacteriologists, or a solution of casein. In the former case the criterion for the occurrence of digestion is the formation of a dimple on the surface of the serum; in the latter, the non-appearance of a cloud, due to the precipitation of casein, on the addition of acetic acid.

Practical Application.—(The antitrypsin of the blood is most markedly increased in cancer. The method has been used chiefly for cancer diagnosis.—*Trans.*)

Method of Marcus[51] (Modification of the Method of Müller and Jochmann)[52].—*Apparatus.*—(1) Trypsin solution: Thoroughly shake in a test-tube 0.1 Gm. trypsin (Kahlbaum) with a mixture consisting of 5 c.c. undiluted glycerine and 5 c.c. distilled water. Then place at 55° for one-half hour, and, after a second thorough shaking, filter. The solution keeps a long time.

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[51] Berlin. klin. Woch. (1908), No. 14, and (1909), No. 4.
[52] Münch. med. Woch. (1909), Nos. 29 and 31.

(2) Loeffler's blood-serum, 11 or 12 dishes. The mixture of 3 parts sterile beef or sheep's serum and 1 part bouillon, made up to contain 1 per cent. peptone, 0.5 per cent. sodium chloride, and 1 per cent. dextrose, is poured into sterile Petri dishes, and solidified at 90° to 95° C. The plates cannot be used immediately after they are prepared, but must stand for several days at room temperature till they have lost their water of condensation and have acquired a firm, elastic surface.

(3) Blood or serum of the patient, obtained three or four hours after the last meal. A few drops suffice.

(4) An incubator at 37°.

(5) Small watch-glasses or hollow ground slides in which to receive the blood, and to prepare the mixture of serum and trypsin.

(6) A platinum loop.

Technique.—Serum or whole blood may be used equally well for the test. If whole blood is used, a couple of drops are received on a hollow ground slide, and may be applied immediately to the Loeffler serum. For this purpose one prepares various mixtures of trypsin solution and of serum or blood. For example, 1 loop of serum or blood with ½, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 loops of the trypsin solution. From each of these mixtures, which are prepared in watch-glasses or hollow slides, 6 or 8 loops are placed on the surface

of the serum in such a way that they will not become confluent. One Petri dish receives trypsin solution only, without blood or serum.

All plates are then placed in the incubator, where they remain for five hours. At the end of this time there will be found, wherever the trypsin has not been completely paralyzed by the blood, shallow dimples on the surface of the Loeffler medium. If, for example, dimple formation occurs with mixtures containing 1 part blood to 3 parts trypsin, but is lacking with mixtures containing 1 part blood to 2 parts trypsin, the blood is said to have a titre of 3:1. By comparing the titre thus obtained with that of normal blood, one determines whether in the given case the antitryptic power of the blood is normal, decreased, or increased. (The greater the amount of trypsin that is required to cause dimple formation, the higher the titre, or, in other words, the greater is the antitryptic power of the blood.)

Method of Bergmann and Meyer.[53]—*Apparatus.*—(1) A 1 per mille solution of trypsin sicc. (Gruebler): dissolve 0.5 Gm. trypsin in 50 c.c. physiological salt solution, add 0.5 c.c. normal soda solution, and make up to 500 c.c.

(2) Casein solution: 1 Gm. casein (Rhenania) is dissolved in 100 c.c. n/10 NaOH solution, with

[53] Berl. klin. Woch. (1908), No. 37.

the aid of gentle heat. Neutralize to litmus with n/10 HCl solution and make up to 500 c.c.

(3) Acetic acid solution: 5 c.c. acetic acid, 45 c.c. alcohol, and 50 c.c. water.

(4) The patient's serum, diluted 20 times with salt solution. 0.1 to 0.2 c.c. suffices.

(5) Small test-tubes.

(6) Pipettes, 2 c.c. in 1–10's.

Technique.—(*a*) *Titration of the trypsin solution.* This must precede the test proper: In each of several test-tubes place 2 c.c. of the casein solution and decreasing amounts of the trypsin solution, as, for example, 1.0, 0.9, 0.8, 0.7, . . . 0.2. Shake carefully and place in the incubator for one-half hour. Then add the acetic acid solution drop by drop, observing which tube shows cloudiness after a few minutes. The tube containing the smallest amount of trypsin, and which remains perfectly clear, contains the "completely digesting dose," which is used for the test proper.

(*b*) *Estimation of the antitrypsin:* In each of 6 tubes place 0.2 c.c. of the patient's serum, and increasing amounts of the trypsin solution, beginning with the completely digesting dose, and increasing by 0.1 c.c. Add 2 c.c. casein solution to each tube and bring all the tubes to a like volume. Incubate as before for one-half hour at 37°. Acidify as before and again note the tube containing the smallest amount of trypsin in which cloudiness

can be seen. The amount of trypsin paralyzed by the antitrypsin of the serum is thus determined. If, for example, the completely digesting dose is 0.7 c.c., and if, in the presence of serum, 2.0 c.c. of trypsin are required to bring about complete digestion, then:

$$2.0 \text{ c.c.} - 0.7 \text{ c.c.} = 1.3 \text{ c.c.},$$

or the amount of trypsin paralyzed by the amount of serum used (in this case 0.2 c.c. of a 5 per cent. dilution, or 0.01 c.c. whole serum). One c.c. of serum, therefore, paralyzes 130 c.c. of the trypsin solution. Fürst [54] expresses the result as follows:

$$\text{Antitrypsin} \frac{37^\circ}{\frac{1}{2} \text{ hour}} = 130.$$

Comparison of the values thus obtained with those given by normal serum shows whether the antitryptic power of the serum tested is altered.

The Meiostagmin Reaction.

Method of Ascoli and Izar.[55]—*Principle.*—When the suitably diluted blood-serum of patients suffering with certain diseases is mixed with the homologous antigen and heated, there occurs a

[54] Berl. klin. Woch. (1909), No. 2.

[55] Ascoli, Ascoli and Izar, and Izar, Münch. med. Woch., vol. 57 (1910), pp. 62, 182, 392, 403, 842, 1170, 2129.

specific lowering of the surface tension of the serum, as measured by the stalagmometer of Traube.

Practical Applications.—The method has been used for the diagnosis of typhoid fever, tuberculosis, syphilis, ankylostomiasis, echinococcus disease, and cancer.

Apparatus.—(1) *A solution of the homologous antigen.* For typhoid, a solution of the products of autolysis of the typhoid bacillus is employed, prepared according to the method of Neisser and Shiga,[56] except that the heating to 60° is omitted. Wash off a 24-hour agar culture of the typhoid bacillus with 10 c.c. sterile physiological salt solution, cover the emulsion with toluol, and shake vigorously. Place in the incubator at 37° for 48 hours, and then filter through a germ-proof filter. The filtrate may be used as antigen; or, better, an alcoholic extract may be prepared in one or other of the two following ways: (*a*) Dry 5 c.c. of the filtrate over a water-bath at 50°. To the dry residue add 10 c.c. of absolute alcohol, shake thoroughly, and centrifugate. To the residue add 10 c.c. of absolute alcohol, shake, and again centrifugate. Combine the centrifugates, and evaporate on the water-bath at 50° to 5 c.c. Or (*b*), to 5 c.c. of the original aqueous filtrate add 20 c.c.

[56] Deutsch. med. Woch., vol. 29 (1903), 61.

of absolute alcohol. Allow the mixture to stand for one-half hour, centrifugate, and mix the sediment with a fresh portion of absolute alcohol, 10 c.c., shake thoroughly, and again centrifugate. Combine the two extracts, and concentrate on the water-bath to 5 c.c.

The syphilis antigen is prepared by extracting 0.5 gramme of a dried and powdered syphilitic spleen with 50 c.c. of absolute alcohol for two hours at 37°, with frequent shaking. Filter, and concentrate to 10 c.c.

To prepare the tuberculosis antigen, proceed as follows: Rub up a (bouillon or agar) culture of the tubercle bacillus in a mortar, and digest for several days at 37° with 95 per cent. alcohol, repeatedly renewed till the alcohol remains clear. Dry the bacillary residue at 47°, and extract with warm ether. Dry, and again extract with 95 per cent. alcohol, renewing the latter as above till it remains clear. Combine the alcoholic extracts, filter, and evaporate to dryness at 47°. Now pour the ether extract into the same evaporating dish which contains the residue of the alcoholic extract, and evaporate to dryness at 30°. Dissolve the entire residue in absolute alcohol, filter, and evaporate at 47° till a sediment begins to form, and again filter. Now add ether, drop by drop, till (having added about 1–10 the volume) a precipitate forms. Filter through thick

filter paper (Dreverhoff No. 41), drive off the ether, and repeat this process till no precipitate forms on the addition of ether. Again evaporate to dryness, dissolve in absolute alcohol, and concentrate to the saturation point.

To prepare the cancer antigen, the undegenerated portions of the tumor are ground in a meat-chopper, spread in a thin layer on glass plates, and dried in the incubator or *in vacuo*. Powder the dry residue in a mortar, and extract for 24 hours at 50° with methyl alcohol, using 25 c.c. for each 5 grammes of tumor tissue. The flask should be shaken occasionally. Filter hot, allow the filtrate to cool to room temperature, and again filter through S. & S. No. 590. *Dry the residue,* and repeat the process, after which it is again dried and again extracted with methyl spirit. Again dry, and extract with warm ether, renewed several times in the course of 24 hours. Dry, and repeat the extraction with methyl alcohol several times till the alcohol remains colorless. Evaporate the alcoholic and ethereal extracts at 50° and at 37°, respectively. A yellowish-red, sticky mass results. Dissolve this in a large amount of water-free ether. Filter, and evaporate at room temperature till a powdery precipitate, slight in amount, forms. This solution constitutes the stock antigen, which, however, may have to be still further concentrated. Too dilute a solution keeps

poorly, while a too concentrated solution emulsifies badly.

Köhler and Luger [57] obtained a more stable and equally effective cancer antigen as follows: Grind up some lecithin (ovolecithin Merck, lecithin Agfa, or lecithin Richter), and extract it for 24 hours at 50° with acetone. Filter clear through S. & S. filter paper No. 590. Just before being used it is diluted with water in such a manner that 1 c.c. contains the largest amount which does not cause a marked reduction of surface tension in normal serum. As a rule, this dilution will be found to lie between 1–60 and 1–100.

K. and L. place the normal variation at from three to eight fractions.

A reaction is positive when the increase ranges from 24 fractions of a drop (1 drop contained 26 fractions with the apparatus used) to 1.5 drops.

The ankylostoma antigen is prepared by rubbing up the worms obtained from feces in a mortar, and extracting to exhaustion with 95 per cent. alcohol at 37°. The residue is dried, powdered, and again extracted. The extracts are combined, dried, and dissolved in absolute alcohol, and filtered.

The fluid contents of an echinococcus cyst may be used as an antigen for the diagnosis of this

[57] Wien. klin. Woch., vol. 25 (1912), 1114.

condition, but this gives less clearly defined results than an antigen prepared from the cyst wall and contents, according to the method given for the ankylostoma.

All these stock solutions keep well, but not indefinitely. They should be kept protected from light, but not necessarily in the ice-box. Shaking seems to injure them. Whenever portions are removed for use, an *absolutely dry* pipette should be employed.

(2) Traube's stalagmometer. Ascoli and Izar use the size giving 56.15 drops of distilled water at 20°. It can be obtained from the Kny-Scherer Co., New York.

(3) Serum of the patient, and of known positive and negative controls.

(4) Test-tubes of the standard size.

(5) Physiological salt solution and distilled water.

(6) Volumetric pipettes, 1 c.c. in 1–100's and 10 c.c. in 1–10's.

(7) Incubator at 37°.

Technique.—The first step is the mastery of the instrument. One should practise until successive readings with the same fluid can be obtained which do not vary more than one or two fractions of a drop. The cleaning of the apparatus is important. It should be washed with distilled water, then 10 per cent. caustic soda solution,

then with distilled water, alcohol, and finally ether. Just before it is used it is again washed with distilled water, and finally rinsed twice with the fluid to be tested. The lower end of the instrument, on which the drop forms, must not be touched with the fingers, and care must be taken to see that the drop forms on the whole of this surface. (When in use, the instrument should be mounted in a clamp or holder, and jarring from heavy footsteps, closing of doors, etc., must be carefully avoided.—*Trans.*)

The standardization of the antigen follows: The stock solution is diluted in varying proportions with 0.85 per cent. salt solution or with distilled water. For the test proper, employ the greatest concentration of the antigen which does not cause an increase of more than one drop, or only slightly more than one drop, when mixed with suitably diluted normal serum and incubated for two hours at 37°. The test may be carried out somewhat as follows: With a *dry* pipette place 0.05 c.c., 0.1 c.c., and 0.2 c.c. of the stock antigen at the bottom of *dry* test-tubes, add rapidly to each 10 c.c. of distilled water and shake vigorously. Now place in each of four test-tubes 9 c.c. of normal blood-serum, diluted 1–20 with salt solution. The blood-serum may be diluted in other proportions, even as high as 1–100, if only a small quantity is available. To one tube add 1 c.c. of distilled water, and to the others 1 c.c. of one of the three

antigen dilutions, and shake all tubes thoroughly. Count the number of drops at once, and place in the incubator in open tubes at 37° for two hours, or in a water-bath at 50° for one hour. When a water-bath at 50° is used, distilled water must be added afterward to make up the loss by evaporation. Allow all tubes to cool to room temperature, and repeat the count. The beginner at least should make all counts in duplicate.[58] Two experiments condensed from Ascoli and Izar, on typhoid and tuberculosis serum respectively, are reproduced below:

TYPHOID SERUM.

Patient's serum 1–10.	Before	After	Normal serum 1–10.	
			Before	After
	Incubation.		Incubation.	
9 c.c.+1 c.c. salt solution.....	57	58	57+1	58
	57+1	58	57+2	58+1
9 c.c.+1 c.c. antigen 1–1000....	58	61+2	57+1	58+2
	58+1	61+1	57	58
9 c.c.+1 c.c. antigen 1–10,000...	58+2	60	57+1	58
	58+1	60	57+1	58
9 c.c.+1 c.c. antigen 1–100,000..	58	59+1	57+1	58
	57+9	59+2	57	57+9

The optimum dilution is 1–1000 or less. A fresh series of dilutions can be made to determine this. The table also illustrates a positive reaction.

[58] An electric device, which rings a bell at the fiftieth drop, can be obtained if desired.

TUBERCULOSIS SERUM.

Patient's serum 1–20.	Before	After	Normal serum 1–20.	
			Before	After
	Incubation.		Incubation.	
9 c.c.+1 c.c. salt solution.....	57+4	58−2	58+3	58+5
9 c.c.+1 c.c. antigen 1–20.....	59	59+5	59+5	60−2
9 c.c.+1 c.c. antigen 1–100.....	57+5	60−2	58+4	58+5
9 c.c.+1 c.c. antigen 1–250.....	57+3	60−5	58+2	58+4
9 c.c.+1 c.c. antigen 1–50.....	59+1	60−4	59+5	60+1
9 c.c.+1 c.c. antigen 1–60.....	59	60−2	60−3	60+2
9 c.c.+1 c.c. antigen 1–70.....	59−4	60−1	59+1	60−4
9 c.c.+1 c.c. antigen 1–80.....	58+1	60−1	58+4	59+1
9 c.c.+1 c.c. antigen 1–90.....	58−3	60−3	58+5	59−3
9 c.c.+1 c.c. antigen 1–100.....	57+5	60−1	58+3	58+4

Although the lower dilution, 1–50 to 1–70, did not cause an excessive increase in the normal serum, it failed to show an adequate increase in the known positive serum. The dilution selected for use was, therefore, one between 1–80 and 1–100. The figures after the plus and minus signs in the tables indicate fractions of a drop as indicated by the calibration on the stem of the instrument. The number of such fractions to a drop will, of course, vary with the size of a drop.

The test proper is carried out in essentially the same manner as is the preliminary titration of the antigen. Dilute 1 c.c. of the patient's serum (Izar has obtained satisfactory results with 15 or 20 drops of whole blood diluted with distilled water,

and also with blood-clots dried on glazed paper, redissolved in distilled water and filtered through cotton) of known normal serum and, when obtainable, of known positive serum with 19 c.c. of 0.85 per cent. salt solution. To 9 c.c. of each dilution add 1 c.c. of the antigen dilution as previously titrated, and of distilled water, respectively. Count the drops at once, and again after incubation. An increase amounting to two drops or more constitutes a positive reaction. The increase in tuberculosis is usually less than in any of the other conditions in which the test has been used. In cancer an increase amounting to four to six drops often occurs.

An extensive literature has accumulated, and the value of the test in diagnosis has been abundantly confirmed. The method is, however, extremely susceptible to errors arising from faulty technique, and, unless all the precautions indicated are observed rigidly, the results are worthless.

Phagocytic Reactions.

Estimation of the Opsonic Index of a Serum According to the Method of Wright.

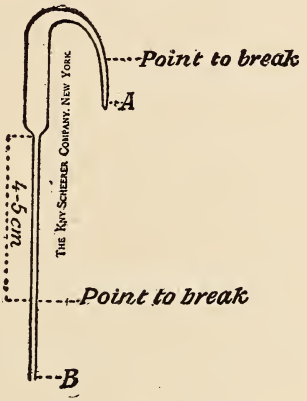
Method of Bine and Lissner.[59]—*Principle.*—When leucocytes are mixed with bacteria and an opsonizing serum, phagocytosis takes place, the

[59] Münch. med. Woch. (1907).

degree of which can be estimated by counting the number of bacteria taken up.

Apparatus.—(*a*) *The patient's serum.* Wright obtains the serum by means of a small glass tube

Fig. 6.



of the form shown in Fig. 6. After swinging the left arm vigorously, the proximal phalanx of the thumb is quickly bound with a small bandage. Break off both ends of the glass tube, and draw up the blood through the end *A*. Seal the opposite end *B* first, scrupulously avoiding heating the blood. When the cooling of the sealed end has drawn the blood away from the end *A*, the latter can also be sealed. After the blood has coagulated, the tube can be hung by its bent end on the rim of a centrifuge tube and centrifugated to complete the separation of the clot. The bent end is

then cut off, and the serum withdrawn with a capillary pipette.

The serum can, of course, be obtained in any other convenient manner.

(*b*) *The serum of normal persons,* similarly obtained, to serve as controls.

(*c*) *Washed blood-cells.* Place a few drops of blood from a normal person in a clean test-tube, two-thirds filled with a freshly prepared 1.5 per cent. sodium citrate solution. Mix thoroughly, but without shaking, and centrifugate. Pipette off the clear fluid, and replace with 0.85 per cent. sodium chloride, and again centrifugate. Remove nearly all of the salt solution, and mix the blood-cells with the remainder. This constitutes the blood suspension ready for use. The cells change after standing only a few hours, so that a fresh suspension must be prepared for each test.

(*d*) *An emulsion of the species of bacteria* against which the blood is to be tested.

The method of preparing this emulsion varies with the species to be used. For tests on tuberculosis it is recommended to use the dry, dead bacteria such as are obtainable from the Hoechst Farbwerke. Grind up a small quantity of this preparation to a fine powder in an agate mortar, and make up to a thick emulsion by adding 1.5 per cent. sodium chloride solution, drop by drop. Sterilize by heating for one hour at 60°. It is most easily preserved in a glass tube, the lower end of

which has been drawn out thin. At the best, it keeps for about 10 days. A few coarse clumps of bacteria are apt to form at the thin, lower end of the tube, and these can be removed by cutting off the end of the tube and resealing.

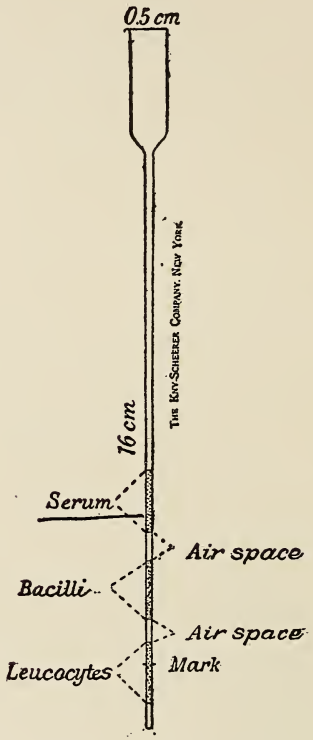
In the case of other species, one loop of a 12- to 24-hour agar culture is usually employed, rubbed up with a little physiological salt solution, and then diluted with more of the same fluid. Strubell [60] recommends, in the case of the colon group and the Gram-negative cocci, cultures only four to ten hours old. The diluting fluid for the tubercle bacillus and the Gram-negative cocci should be a 1.5 per cent. solution of sodium chloride. Emulsions of streptococcus have to be shaken very vigorously to render them homogeneous. To remove chains or threads remaining in the emulsion, the latter may be centrifugated a short time, and the upper part carefully poured from the sediment.

Before it is used, the emulsion must be tested to determine whether it contains a sufficient number of bacteria. According to Wright, the number should be about 7000 to 10,000 million in 1 c.c. The number can be readily estimated by mixing together equal volumes of blood and emulsion, smearing a drop of the mixture on a slide, fixing, and staining. Since 1 c.c. of blood contains about 5000 million red cells, a field should average two

[60] Deutsch. med. Woch. (1908), No. 19.

bacteria to one red cell. If the number is greater than this, the emulsion should be diluted accordingly.

Fig. 7.



(*e*) *Capillary tubes* made by drawing out glass tubing of about 5 mm. inside diameter over the gas flame. The capillary part should be 16 cm. long (Fig. 7). The upper wide end is provided with a rubber nipple.

(*f*) Slides, thoroughly cleaned, sterilized, and *slightly* roughened by rubbing with fine emery paper. Cover-glasses.

(*g*) A so-called spreader, that is, a slide with the edge ground out slightly concave, to use in making the smear.

(*h*) Saturated solution of mercury bichloride for use as a fixative.

(*i*) Ziehl-Nielson carbofuchsin or carbothionin for the tubercle bacillus, eosin methylene blue, May-Grünwald (or Wright's, *Trans.*), etc.

(*j*) Microscope with homogeneous immersion lens.

(*k*) An incubator at 37°.

(*l*) A Bunsen burner.

(*m*) A glass knife or file.

Technique.—Make a mark 2 or 3 cm. from the end of the capillary tube. With the rubber nipple draw up the serum to this mark; then admit a bubble of air; then the blood suspension to the mark; then another bubble; then the bacterial suspension to the mark. The capillary now contains equal volumes of the three fluids, separated from one another by little bubbles of air. (Fig. 7.) The capillary is immediately emptied on a slide, or, perhaps better, on the bottom of a short test-tube, the fluids mixed as quickly and thoroughly as possible, and again drawn into the capillary and the end sealed. Heating of the contents must,

of course, be avoided. The same treatment is given the control tube containing the normal serum.

Now place the sealed tubes for 20 to 30 minutes in the incubator. After incubation, cut off the end of the tubes and smear the contents of each on a slide, using the "spreader," and avoiding the use of any pressure. Saathoff prefers to place the contents of the tubes on slides, and to mix thoroughly with a platinum loop.

After the slides become air-dry, fix for two or three minutes in the sublimate solution, and then stain.

One next proceeds to count the number of bacteria contained in each leucocyte. At least 100 leucocytes must be counted. Experience has shown that the largest number of cells is found in the edge of the smear, where the latter is thinnest. The number should range from two to eight bacteria to the cell. If more or fewer are present, the counting becomes more difficult in the one case and inaccurate in the other. The average number of bacteria per cell constitutes the phagocytic count. The average value given by at least three normal individuals should be taken as the normal. In order to be sure that an equally thick emulsion of bacteria is used for each test, Bine and Lissner recommend that before an emulsion is used a count should be made with a normal (one's

own) serum. This should give always the same value.

The phagocytic count of the patient's serum, divided by the normal count, gives the opsonic index of the patient.

Slight deviations from the technique above described affect the result to a marked extent, and render the results unreliable.

The chief use of the opsonic index in clinical medicine has been in the estimation of the stage or progress of the disease in infectious processes, and for the purpose of making continuous controls in the use of therapeutic agents, especially of Wright's vaccines. It may, however, serve for purely diagnostic purposes. Sauerbeck[61] lays down the following rules for use with this object in view:

When the opsonic index for a certain species of bacteria is above normal, infection with this species is excluded. If the index is permanently lowered, the process is a local one. When it is permanently raised, the infection has been overcome, or an artificial (immunizing) infection has progressed to a certain point. According to Schottmüller and Much,[62] the opsonins exhibit a departure from the normal at the very beginning of an infection, and in infections of the lightest

[61] Neue Tatsache und Theorien der Immunitätsforschung, Bergman, Wiesbaden, 1907.

[62] Münch. med. Woch. (1908), No 9.

grade, in which agglutinin is not present in the serum and in which, therefore, the Widal reaction can be of no assistance. Since the immune opsonins are more resistant to heat than the normal opsonins, a comparison of the index obtained before heating with that obtained after heating for 10 minutes at 60° may give useful results. If the difference is marked, it shows that the opsonins present are chiefly normal, and that the opsonins are not changed as the result of the absorption of bacteria. If, on the other hand, the opsonic power of the serum is not much reduced by heating, infection or immunization, as the case may be, may be inferred. Experience with this diagnostic method is still very limited.

Method of Kammerer.[63]—*Apparatus.*—(1) *Serum* of the *patient* and of a *healthy person,* obtained in the usual manner, and not heated (not inactivated).

(2) *A bacillary emulsion* in physiological salt solution containing 1.5 per cent. sodium citrate.

(3) *Blood of a healthy person,* drawn from the finger-tip directly into the Wright capillary pipette, described above (*e*), p. 132.

(4) The remaining apparatus is as described above under (*f*) to (*m*), p. 133.

Technique.—Fill the capillary tube to the mark with blood of a normal person, then air, then blood

[63] Münch. med. Woch. (1908), No. 20.

again, then air, then patient's serum, then air, then, finally, bacillary emulsion. In a second capillary, blood, air, blood, air, normal serum, air, bacillary emulsion.

Mixing and further handling are as described on p. 133 *et seq.* *Rapid work is necessary.* After sealing, the capillaries are placed for one-half hour in the incubator. Prepare the smears on cover-glasses and, after staining, mount in Canada balsam, which, on account of its greater transparency, facilitates the counting.

The method is said to have the following advantages:

(1) Simplification of the technique by combining the separate steps of obtaining and washing the leucocytes.

(2) Less injury to the leucocytes, due to washing and centrifugating.

(3) A relatively thick layer of leucocytes, which facilitates the count.

(4) The action of the "amboceptor-like immune opsonins" is favored by the presence of complement, producing an increased difference between the normal and the patient's serum.

The above has not been confirmed.

Demonstration of Antistaphylolysin in Serum.—In patients suffering from staphylococcus infection.

Principle.—One determines what amount of

the serum is required to protect rabbit's red blood-cells against solution by staphylolysin.

Apparatus.—(*a*) Two or 3 c.c. *defibrinated rabbit's blood:* The blood from the severed carotid is received in a strong, thick-walled test-tube. containing glass beads to about a finger's depth. Close the tube with a cork or rubber stopper and shake for several minutes. Do not fill the test-tube too full of blood.

If long-eared rabbits are available, the blood can be obtained from the ear vein. To do this, rub the ear briskly with cotton wet in ether until the vessels become distended. Then with a sharp knife sever the vein transversely. As the blood issues from the vein it is caught in a graduated tube containing 2 or 3 c.c. of 1 per cent. sodium citrate. The blood should be well mixed with the citrate solution as soon as it comes in contact with it, to prevent coagulation. Two or 3 c.c. are thus collected, making the whole amount of mixture 4 to 6 c.c.

Remove the citrated plasma from the cells by repeated washing with 10 volumes of physiological salt solution. After the last washing, make the sediment of cells up to the original volume with salt solution.

(*b*) *Staphylolysin:* The most suitable medium for the preparation of staphylolysin is a bouillon containing one-third to one-half the amount of alkali needed to produce a distinct red reaction

with phenolphthalein. This amount is, of course, determined by titrating a small amount of the medium, say 10 c.c., against phenolphthalein, and calculating for the entire volume. One-half to one litre of such a bouillon is inoculated with *Staphylococcus aureus,* after a preliminary test has proved that the strain is able to produce hæmolysis. This test can be made by inoculating several tubes containing 2 c.c. of bouillon with the staphylococcus strain. Beginning at the third or fourth day, one tube is tested each day by adding a drop of defibrinated rabbit's blood and placing for two hours in the incubator. If marked hæmolysis occurs, as shown after sedimentation for 18 hours in the ice-box, or after centrifugation, the strain is suitable for the purpose of the test.

After the flask culture has grown for 13 or 14 days, it is filtered germ-free through a Berkefeldt filter and 0.5 per cent. carbolic acid is added. Preserve in the ice-box.

(*c*) The *patient's serum,* inactivated by heating for one-half hour at 56°.

(*d*) Physiological salt solution.

(*e*) Small test-tubes, 1 cm. in diameter by 10 cm. long.

(*f*) Volumetric pipettes, 1 c.c. and 10 c.c. in 1–10's and 1–100's.

(*g*) *Normal horse serum* dried *in vacuo,* as standard serum. Dissolve fresh for each test in 10 volumes sterile distilled water.

Technique.—Two separate determinations are involved:

(1) The estimation of the activity of the staphylolysin.

(2) The estimation of the antagonistic activity of the patient's serum.

For the first, place in a series of test tubes the following amounts of staphylolysin:

1.0 0.75 0.5 0.25 0.1

and of a 10 per cent. dilution of the same, made with salt solution (1 c.c. lysin + 9 c.c. salt solution):

0.75	0.5	0.25	0.1
(=0.075)	(=0.05)	(=0.025)	(=0.01)

and finally of a 1 per cent. dilution (1 c.c. of the 10 per cent. dilution + 9 c.c. salt solution):

0.75	0.5	0.25	0.1
(=0.0075)	(=0.005)	(=0.0025)	(=0.001)

Make up all tubes to 2 c.c. with salt solution, add 1 drop of rabbit's blood, and mix. Place the tubes in the incubator at 37° for two hours, and, having noted the results, leave them over night in the ice-box. Reëxamine the next morning. Usually hæmolysis is only slightly more advanced than after two hours in the incubator.

The amount of staphylolysin required to effect complete hæmolysis is made the basis of the second part of the test.

For the second portion, place the dissolving dose of staphylolysin as above determined in a series of tubes, and add the following amounts of the serum to be tested:

1.0	0.75	0.5	0.25	0.1	
	0.075	0.05	0.025	0.01	(added in the form of a 10 per cent. dilution of the serum).
	0.0075	0.005	0.0025	0.001	(added in the form of a 1 per cent. dilution of the serum).

Bring all tubes to 2 c.c. with salt solution, add 1 drop of rabbit's blood, and treat as above. Note the volume of serum which completely neutralizes the hæmolytic effect, as indicated by the fact that the supernatant fluid above the blood-cells remains entirely uncolored.

In order to have a basis for comparison of the antihæmolytic activity of the patient's serum at various times, Neisser and Wechsberg recommend, in view of the possible deterioration of the staphylolysin, that a simultaneous determination of the inhibiting power of normal horse serum be always made, the latter to be preserved in the dry form, and therefore not subject to deterioration. The comparison of the two values (that of the patient's serum with that of the dry horse serum) gives the relative activity of the serum tested, as compared with the constant. This value is independent of the staphylolysin used.

According to Arndt [64] and others, a high antistaphylolysin content of the serum is always a sign of staphylococcus infection, even if it is impossible to establish fixed limits for the values.

Determination of Diphtheria Toxin in the Blood Serum (Uffenheimer's Method).[65]—*Principle.*—Small amounts of diphtheria toxin, too slight to cause death of a laboratory animal (guinea-pig), may, nevertheless, cause, after 24 hours, distinct œdema or infiltration at the seat of injection into the same animal.

Apparatus.—(*a*) 0.1 to 0.3 c.c. serum from the patient.

(*b*) A guinea-pig weighing about 250 grammes.

(*c*) Physiological salt solution, to dilute the serum.

(*d*) A sterilizable syringe of 2 to 4 c.c. capacity.

(*e*) A sterile sedimentation glass, in which to mix the serum and salt solution.

(*f*) Operating table and instruments.

Technique.—The serum to be injected is made up to 2 to 4 c.c. with salt solution, and injected into the guinea-pig intraperitoneally (*not intramuscularly!*). After 17 to 20 hours one can feel a very distinct doughy œdema of the abdominal cavity. At the end of 48 hours kill the animal by a blow on

[64] Deutsch. med. Woch., 1907.

[65] Münch. med. Woch. 3 (1906), and (1907), No. 3.

the back of the neck, place it on the operating table, and open the thorax and abdomen. The œdema can then be seen readily.

The diagnostic value of this method has not yet been adequately established. On the one hand, Uffenheimer maintains that the diagnosis of true diphtheria can often be made more quickly by this method than by bacteriological examination; on the other hand, it is true that the reaction is not always present in known diphtheria.

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